Molecular Mechanisms of Heavy Metal Homeostasis in Flies and Humans

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Zusammenfassung

Ionen von essentiellen Schwermetallen, wie Zink und Kupfer, spielen eine wichtige Rolle für die Struktur und Funktion vieler zellulärer Proteine. Sind diese Schwermetalle in unzureichender Menge im Organismus vorhanden, so kommt es zu schwerwiegenden Mangelerscheinungen, die sich in einer verminderten Immunfunktion oder einer Wachstumshemmung zeigen können. Andererseits ist ein Überschuss dieser Schwermetalle schädlich. Deshalb ist es nicht verwunderlich, dass alle Organismen einen Weg gefunden haben, die optimale zelluläre Konzentration dieser Ionen zu gewährleisten (Homöostase). Dies wird dadurch erreicht, dass die Expression von spezifischen Importproteinen, Exportproteinen und Metallothioneinen streng reguliert wird. Metallothioneine sind kleine, cysteinreiche Proteine, die Metallionen binden und sie somit unschädlich machen. In den meisten höheren Organismen spielt der Transkriptionsfaktor „metal-responsive transkription factor-1“ (MTF-1) bei der Schwermetallhomöostase der Zelle eine zentrale Rolle. Wird die Zelle erhöhten Konzentrationen an Schwermetallionen, oxidativem Stress oder Hypoxie (unzureichende Sauerstoffversorgung) ausgesetzt, aktiviert MTF-1 die Expression von Metallothioneinen und anderen Proteinen, welche die Zelle vor Schädigungen schützen.

In der vorliegenden Arbeit charakterisieren wir die Funktion eines cysteinreichen Sequenzmotifs, welches im MTF-1 Protein aller Wirbeltiere konserviert ist und somit eine wichtige Funktion besitzen muss. Werden diese Cysteine mutiert, verliert MTF-1 weitgehend seine transkriptionelle Aktivität, kann jedoch noch an DNA binden. Hier wird in einer Reihe von Experimenten gezeigt, dass die Cysteine für die Homodimerisierung von MTF-1 notwendig sind. Die Dimerisierung von MTF-1 kann nicht durch eine Exposition der Zellen mit Zink induziert werden ist somit konstitutiv. Wir gehen davon aus, dass nur die dimere Form von MTF-1 in der Lage ist, Kofaktoren und den allgemeinen Transkriptionsapparat zu den Promotern der von MTF-1 regulierten Gene zu rekrutieren.

Die Interaktion zwischen p300 und hMTF-1 wird im zweiten Teil dieser Arbeit diskutiert. p300 ist eine sogenannte Histon-Acetyltransferase und ist Teil der generellen Transkriptionsmaschinerie. Durch Co-Immunpräzipitationsexperimente

wird gezeigt das p300 und hMTF-1 miteinander interagieren und in einem zellfreien Testsystem kann eine Acetylierung von hMTF-1 durch p300 nachgewiesen werden.

Im dritten Teil dieser Arbeit werden vorläufige Ergebnisse gezeigt, die sich mit der Charakterisierung der funktionellen Domänen des MTF-1 Proteins der Fruchtfliege *Drosophila* (dMTF-1) befassen. Das Ziel ist es, die Proteinsegmente, welche für die transkriptionelle Aktivität, die zelluläre Lokalisation und das Erkennen von zu niedrigen oder zu hohen zellulären Metallkonzentrationen notwendig sind, zu identifizieren. In den letzten Jahren wurde in einer Reihe von Studien die Domänenstruktur des MTF-1 Proteins in Säugetieren untersucht. Eine solch umfassende Studie fehlte für dMTF-1 bisher. Die hier dargestellten Versuchsansätze und vorläufigen Ergebnisse werden zum einen Aufschluss über die Funktionsweise von dMTF-1 geben und zum anderen einen Vergleich mit der Funktionsweise des MTF-1 Proteins der Säugetiere erlauben.

Im letzen Teil untersuchen wir eine Nullmutante des Atox1 Gens im Modellorganismus *Drosophila melanogaster*. Atox1 ist ein sogenanntes Chaperon, welches dafür verantwortlich ist Kupferionen, welche durch die Zellmembran in die Zelle gelangen, durch das Zytoplasma zu den inneren Zellorganellen (Golgi-Apparat) zu transportieren. Von dort wird Kupfer durch Exozytose wieder aus den Zellen hinausbefördert. In den Zellen des Darms ist dieser Transportmechanismus besonders wichtig, da Kupfer auf diesem Weg in den Körper gelangt. Fehlt Atox1, akkumuliert Kupfer in den Darmzellen mit daraus resultierendem Kupfermangel im Rest des Körpers. Fliegen, welche kein Atox1 Chaperon besitzen, können sich im Gegensatz zu Wildtyp-Fliegen nicht entwickeln, wenn wenig Kupfer durch die Nahrung bereitgestellt wird. Andererseits sind diese Fliegen resistent gegen Cisplatin, ein Antikrebsmedikament, welches durch zelluläre Kupfertransportwege in den Organismus gelangt.

Summary

Heavy metal ions, like Zn^{2+} and $Cu^{+/2+}$, are important for the structure and function of many cellular proteins. If an organism is supplied with inadequate amounts of these heavy metals, it is suffering from severe deficiency syndromes, including growth retardation and a compromised immune function. On the other hand, accumulation of these ions is toxic for the organism. For these reasons every organism has to find a way to keep the concentration of essential heavy metals at an optimal level. To achieve this, the expression of metal ion importers, exporters and metallothioneins is tightly regulated. Metallothioneins are small, cysteine-rich proteins that bind metal ions and thereby detoxify the cell. In most higher organisms the metal-responsive transcription factor-1 (MTF-1) plays a central role in protecting the cell from the adverse effects generated by heavy metal ions as it activates the expression of metallothioneins and other cytoprotective proteins.

In this work we characterize the function of a cluster of cysteines, which is conserved in all vertebrate homologs of MTF-1. If these cysteines are mutated, MTF-1 is severely impaired in activating its target genes under normal and especially under metal-induced conditions, but is still able to bind DNA. We can show that these cysteines mediate homodimerization of MTF-1. The dimerization of MTF-1 cannot be boosted by treating cells with zinc and rather seems to be constitutive. We suggest that only the MTF-1 dimer is able to recruit proteins of the general transcriptional machinery to the promoter region of MTF-1 target genes.

The interaction of MTF-1 and p300 will be discussed in the second part of this work. p300 is a histone acetyltransferase and is part of the general transcriptional machinery. Our data demonstrate that MTF-1 is interacting with p300 and is acetylated by p300 in a cell free test system.

The third part deals with the characterization of the functional domains of the *Drosophila* MTF-1 homolog (dMTF-1) and preliminary results will be shown. The aim is to identify the protein segments that mediate transcriptional activity, regulate subcellular localization of the protein, and sense heavy metal concentrations. The experiments and results presented here will elucidate regulatory mechanisms of

dMTF-1 and allow a comparison with mammalian MTF-1, whose domain structure has been studied intensively over the past years.

In the last part we study a *Drosophila melanogaster* mutant, which lacks the Atox1 gene. Atox1 is a so called chaperone which transports copper ions from the plasma membrane to ATP7, a copper exporter. ATP7 delivers copper to proteins located in the trans-Golgi network. From there, copper is exported out of the cell via exocytosis. This route of transport is particularly important in enterocytes, where copper is taken up from the food and is provided to the organism. If Atox1 is missing, copper accumulates in the gut cells, with copper deficiency in the rest of the body as a consequence. Larvae that do not express Atox1 cannot develop under copper starvation conditions that pose no problem for wild type flies. On the other hand, these flies are resistant to the anticancer drug cisplatin, a compound whose uptake and distribution in the body is mediated by copper transport mechanisms.

Introduction

Essential trace metals in biology: zinc and copper

Metals like zinc and copper play an important role in the function and structure of a set of cellular proteins. Zinc is an essential catalytic cofactor of several enzymes and acts as a structural component of numerous proteins. Next to iron, zinc is the most abundant trace element in higher organisms and it is estimated that up to 10 % of all human proteins bind one or more zinc ion (2). Zinc-containing proteins are mainly involved in all steps of gene expression, DNA repair and apoptosis (24). Furthermore, there are more than 300 zinc-containing enzymes, including carboxypeptidase A, alcohol dehydrogenase and carbonic anhydrase (16). Zinc is indispensible for cell differentiation, the defense against oxidative stress and the immune response; clinical symptoms resulting from zinc deficiency include recurrent infections, growth retardation, anorexia, mental lethargy and iron-deficiency anemia (69). The daily dietary intake of zinc is 15 mg/day (83) and deficiency can result from malnutrition, alcoholism, gastrointestinal and renal diseases and acrodermatitis enteropathica, a rare genetic defect in which SLC39A4, the gene encoding the zinc importer ZIP4, is mutated (40). On the other hand, very high doses of zinc induce oxidative stress, mitochondrial dysfunction and apoptosis (94). Organisms are relatively tolerant to high levels of orally administrated zinc, but there are reports that high doses of zinc can interfere with copper and iron uptake and thereby lead to symptoms of copper deficiency and anemia (67, 70).

Copper is an essential co-factor in the active center of cuproenzymes, which rely on the ability of copper to undergo redox cycling between the Cu^{+} and Cu^{2+} state. Examples of copper-containing enzymes are cytochrome c oxidase (CCO), a component of the mitochondrial respiratory chain, Cu,Zn-superoxide dismutase (Cu,Zn-SOD or SOD1), an enzyme essential for free radical defense and tyrosinase, which is involved in melanin synthesis and thereby indispensable for the defense against UV-irradiation. The recommended daily dietary intake of copper ranges between 1.5 and 3 mg (76). Due to its redox activity, copper is toxic if accumulated in excess, as it generates free radicals via the Fenton reaction. Moreover, copper ions

can replace other metal cofactors and thereby block a protein's function. For these reasons, cells keep the free copper ions virtually nonexistent.

Several diseases are characterized by a distorted copper metabolism. The best known examples are Menkes and Wilson Disease. In Menkes disease, the ATP7A gene is mutated, which leads to an inefficient uptake of copper in the intestine (see below). Menkes disease is characterized by symptoms of copper deficiency, which are growth retardation, brain degeneration and altered hair structure (56). The liver specific ATP7B transporter (see below) is mutated in Wilson disease. Thereby, copper accumulates in the brain, the liver and other organs including the eyes, in which copper depositions are visible as the so-called Kayser-Fleischer rings (78). Clinical symptoms include liver failure as well as neurological and psychiatric disorders (1).

Due to their conflictive properties in being essential elements for maintaining cellular functions and being toxic if accumulating in excess, every organism has precise mechanisms to regulate the bioavailability of zinc and copper (13, 17, 58, 71). A coordinated expression of proteins that are involved in import, storage, sequestration and export of these metals ensures that they are maintained at an optimal level inside the cell.

Zinc transport and trafficking

Two protein families are involved in zinc transport. The ZnT (SLC30) family members are responsible for zinc export and the decrease of intracellular zinc levels, whereas the ZIP (Zrt- and Irt-like proteins, SLC39) transporters mediate zinc import from the extracellular space or organelles (17, 47). 10 ZnT transporters (ZnT1-10) have been described for mammals and are characterized by 6 transmembrane domains; ZnT5 even has 12 transmembrane domains. They contain a characteristic glycine-histidine-rich intracellular loop that was suggested to bind zinc. The different members of the ZnT family are present in different organs and intracellular compartments (Figure 1) and their expression is in part regulated by the nutritional status.

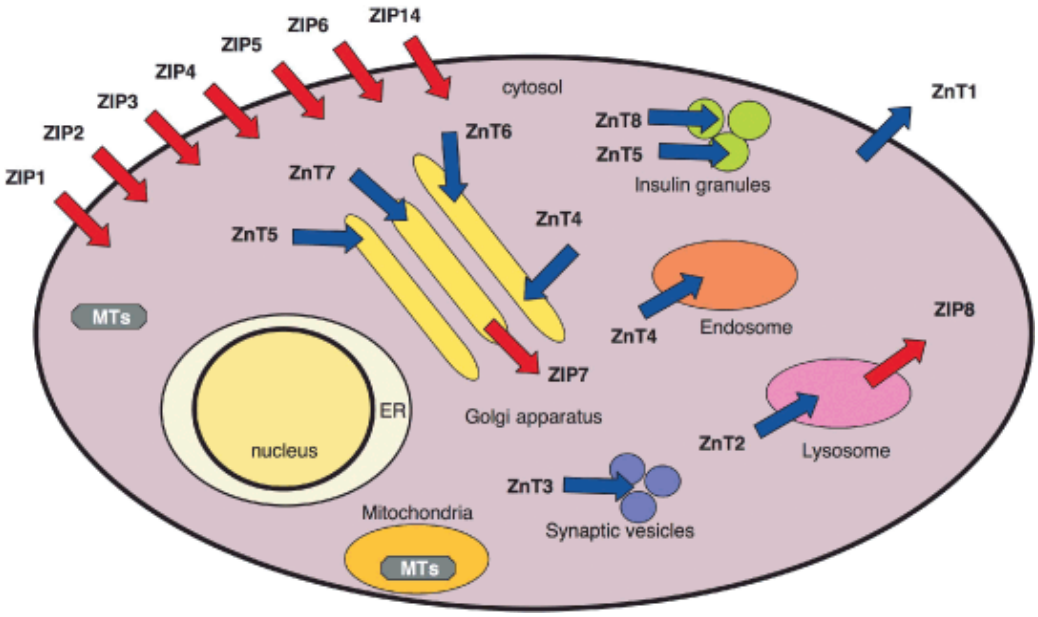

Figure 1: Subcellular localization of characterized ZnT and ZIP family members. ER, endoplasmatic reticulum (Adapted from Murakami and Hirano, 2008)

ZnT-1 is the only family member that is located at the plasma membrane (63). Mutation of ZnT-1, which is the main regulator of cellular zinc efflux, is homozygous lethal and the basal as well as metal-induced expression of ZnT-1 is dependent on the metal-responsive transcription factor-1 (MTF-1) (42). The ZIP family consists of 14 members, most of which have eight transmembrane domains and a histidine-rich intracellular loop (84). Like the ZnT proteins, the ZIP family members are found in different organs and show a diverse subcellular localization (Figure 1 and (47)). Their activity can be regulated at the transcriptional or post-transcriptional level, by changing their subcellular localization depending on the zinc status (52, 89).

In *Drosophila* only three zinc transporters have been characterized. Fear of intimacy (foi) is essential for gonad morphogenesis and glial cell migration and belongs to the ZIP family of zinc transporters (55). Znt35C, which belongs to the ZnT family of zinc transporters, was found in a screen designed to identify genes that are changed in response to zinc, copper and cadmium load and copper starvation in *Drosophila* (96). The fly ortholog of ZnT1 confers zinc transport in enterocytes of the gut and is essential for dietary zinc absorption (90).

Intracellular trafficking of copper

Copper is taken up into the cell by members of the highly conserved family of Ctr (SLC31) copper importers (64). All members have 3 transmembrane domains and a homotrimeric complex forms a channel that allows copper to pass the plasma membrane and enter the cell (44). Two forms have been found in humans, hCtr1 and hCtr2. hCtr1 localizes to the plasma membrane and is the main cellular copper importer (99), whereas hCtr2 is localized to late endosomes/lysosomes and might contribute to copper homeostasis by releasing copper from intracellular pools (88). hCtr1 cycles between the plasma membrane and endosomes and undergoes rapid lysosomal degradation in response to excess copper (30, 66). In *Drosophila* three Ctr genes were described, *Ctr1A*, *Ctr1B* and *Ctr1C* (9). Whereas Ctr1A is constitutively expressed throughout all stages of development, Ctr1B is highly expressed in late embryonic and larval stages. Ctr1B is localized to intestinal cells and its expression is induced upon copper deficiency via dMTF-1 (79, 86, 100).

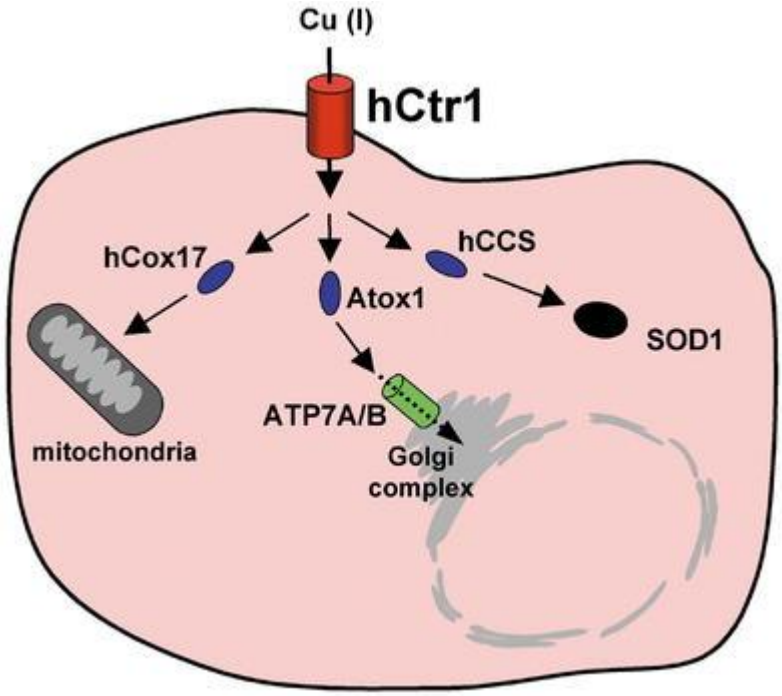

Figure 2: Intracellular copper trafficking pathways. Copper that enters the cell via Ctr1 is distributed to its target proteins by three distinct copper chaperones, Cox17, Atox1 and CCS (adapted from Petris, 2004).

Next to metallothioneins that scavenge excess copper and serve as a copper store especially in *Drosophila* (8), there are three intracellular copper chaperones that mediate copper trafficking to specific cuproenzymes (Figure 2). CCS delivers copper to the cytosolic SOD1 (74). COX17 transfers copper to SCO11 and COX11 that are donors for the Cu_A and Cu_B sites of cytochrome C oxidase, respectively (31). Atox1 (Atx1 in yeast) provides copper to the P-type ATPases located in the *trans*-Golgi-network, whereby copper is delivered to cuproenzymes located in the secretory pathway, or can be excreted (32). In part IV of the present work we characterize a null mutation of the *Drosophila* gene of Atox1, and show that Atox1 mutant flies are sensitive to copper deprivation but show an increased resistance to the anti-cancer drug cisplatin.

Two Cu-P-type ATPases have been identified in humans, ATP7A and B. They have a 67 % protein sequence homology but are expressed in different organs. ATP7A is detected in intestinal cells and in most other tissues except the liver; ATP7B, on the other hand, is mainly expressed in the liver, where it is essential for biliary copper excretion and delivery of copper to ceruloplasmin, the main serum copper protein (41). Both proteins are normally located to the *trans*-Golgi network, but translocate to post-Golgi compartments close to the plasma membrane upon copper load (65, 75) to excrete excess copper from the cell. In *Drosophila* only one ATP7-like transporter was identified, DmATP7, that combines the functions of ATP7A and B (60). Deletion of DmATP7 results in copper accumulation in intestinal cells with concomitant copper deficiency in the rest of the body (7).

The metal-responsive transcription factor-1

A key player in heavy metal homeostasis in higher organisms is the metal-responsive transcription factor-1 (MTF-1), whose main target genes are the metallothioneins. Homologous genes of MTF-1 have been identified and characterized in human (12), mouse (73), the giant rodent capybara (51), the Japanese pufferfish *Fugu rupriens* (5), the common carp (25), zebrafish (21) and the fruitfly *Drosophila melanogaster* (97). Next to its role in heavy metal homeostasis, MTF-1 is an important factor in the defense against oxidative stress (20, 93) and hypoxia (19, 27, 59). The fact that MTF-

1 is shown to be upregulated in tumor cells, which are exposed to oxidative stress and hypoxia due to their rapid proliferation (80), and contributes to the development of gallium-resistant tumors (95) further underlines its central role in the protection of cells against cellular damage caused by diverse stressors.

MTF-1 knockout mice are not viable and die during embryonic development on day 14 of gestation due to hepatocyte necrosis (29). A conditional knockout showed that adult MTF-1 -/- mice are viable, but are highly sensitivity to cadmium exposure and have defects in hematopoiesis (91). In contrast to this, a *Drosophila* knockout mutant of MTF-1develops with indistinguishable rate and is viable under normal laboratory conditions (22). If exposed to heavy metal load with cadmium, copper and zinc, MTF-1 knockout flies fail to develop as they are unable to activate detoxification systems.

MTF-1 target genes

The best characterized target genes of MTF-1 are those coding for metallothioneins (MTs). MTs are small (< 7 kDa), cysteine-rich proteins (30 %) that protect cells against heavy metal intoxication and oxidative stress (18, 85). MTs bind up to 7 divalent ions and 18 different metal ions can be incorporated. In mammals there are four classes of MT genes. MT1 and MT2 are target genes of MTF-1 and are ubiquitously expressed under diverse stress situations. MT3, which is not regulated by MTF-1, is a brain specific gene and MT4 expression is restricted to the squamous epithelium and the maternal deciduum. In humans, 10 functional MT isoforms have been identified, including 7 MT-1 isoforms and one isoform each of MT-2, -3 and -4. Mice which lack MT-1 and MT-2 (also referred as MT-IIA) are viable, but sensitive to elevated concentrations of heavy metals and inflammation (54, 57). In *Drosophila*, five metallothionein genes have been found, designated as MtnA – E ((22) and unpublished). A knockout of metallothionein A – D is highly sensitive to elevated concentrations of cadmium and copper (23).

γ-GCS_{hc}, the gene that encodes the heavy-chain subunit of the gamma-glutamylcysteine synthetase, a key enzyme for glutathione biosynthesis, was suggested to be a target gene of MTF-1 as MTF-1 knockout mouse embryos show

reduced transcript levels of this gene (29). Later, alpha-fetoprotein, C/EBPalpha and tear lipocalin were identified as target genes using MTF-1 knockout mouse embryos (49). Furthermore, MTF-1 is required for the basal expression of selenoprotein W, muscle 1 gene (Sepw1), which is involved in redox homeostasis. MTF-1 also mediates the cadmium-induced expression of N-myc downstream regulated gene 1 (Ndrg1), which has anti-metastatic properties and is up-regulated in response to diverse stress signals, and cysteine- and glycine-rich protein 1 gene (Csrp1), which functions in cell differentiation and development. MTF-1 is also involved in the repression of Slc39a10, a putative zinc transporter (93). In response to zinc and cadmium, MTF-1 also activates the expression of ZnT-1, a zinc exporter (42). Kruppel-like factor 4, hepatitis A virus cellular receptor 1 and complement factor B were found as potential MTF-1 target genes in an assay using MTF-1 overexpression (38). Prion proteins were suggested to have a role in copper homeostasis and together with Sp1, MTF-1 was reported to be involved in prion protein gene activation (10).

Like in mammals, *Drosophila* MTF-1 is essential for the basal and metal-induced expression of all metallothionein genes (22). dMTF-1 is also active under copper starvation where it mediates expression of the intestinal copper importer Ctr1B (79). Using MTF-1 knockout flies, ferritins, the ABC transporter CG10505 and the zinc transporter ZnT35C were identified as dMTF-1 target genes (96).

Regulation and functional domains of mammalian MTF-1

Transcripts of the MTF-1 gene are hardly, if at all, increased after metal treatment (6), suggesting that its function is mainly regulated translationally or posttranslationally. MTF-1's activity could be regulated at several steps: the nuclear-cytoplasmic shuttling, the DNA binding, and finally the interaction with transcriptional co-activators leading to the activation of target genes.

The DNA-binding domain of MTF-1, consisting of six zinc fingers, recognizes a specific DNA sequence motif TGCRCNC, which is called the metal response element (MRE). The MTF-1 target genes typically contain several of such MREs in their enhancer/promoter regions. Three C-terminal activation domains were described for

mammalian MTF-1: an acidic, a proline-rich, and a serine-threonine-rich domain, with the acidic activation domain being the strongest (72).

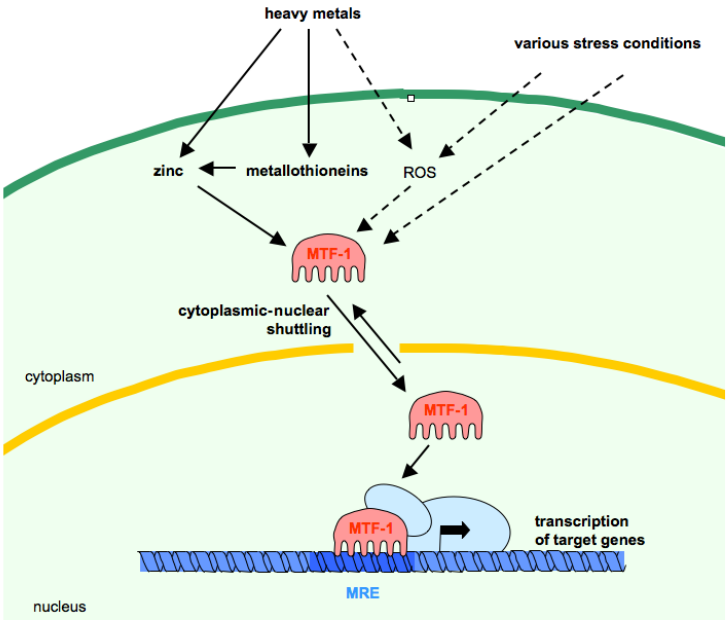

Figure 3: Overview of MTF-1 regulation. Under normal conditions, MTF-1 mostly resides in the cytoplasm, but translocates to the nucleus under different stress conditions like heavy metal load, oxidative stress and hypoxia. MTF-1 target genes, such as those encoding metallothioneins, typically contain several MTF-1 binding sites, called MREs (metal-response elements), in their enhancer/promoter region.

Under normal conditions MTF-1 mainly resides in the cytoplasm, but rapidly translocates to the nucleus in response to metal exposure and other stress signals (Figure 3 and (77, 81). A conventional nuclear localization signal located N-terminal of zinc finger 1 was identified by sequence comparison, but mutation of this motif only had minor effects on nuclear translocation (77). A more potent, but non-conventional nuclear localization signal overlaps with zinc fingers 1-3 (50). Nuclear export of MTF-1 is accomplished by a nuclear export signal which overlaps with the acidic activation domain (50). The importance of nuclear export is unclear, as a constantly nuclear mutant human MTF-1 performs close to wild type regarding basal and induced metallothionein expression.

It has been proposed that MTF-1 serves as an intracellular zinc sensor itself (3, 48), but the molecular mechanisms are not yet understood. Two domains can fulfill this

function: The zinc fingers have diverse zinc-binding affinities (28, 68), and MTF-1 shows an increased DNA-binding activity *in vitro* upon zinc treatment (34, 62). Recently it was also suggested that the zinc-finger linker region is involved in sensing metals (46). The acidic activation domain was previously shown to act as an independent, zinc-responsive activation domain in a subset of cell lines tested (50). Induction of MTF-1 target gene expression by other activators than zinc may work via release of zinc from metallothioneins (98).

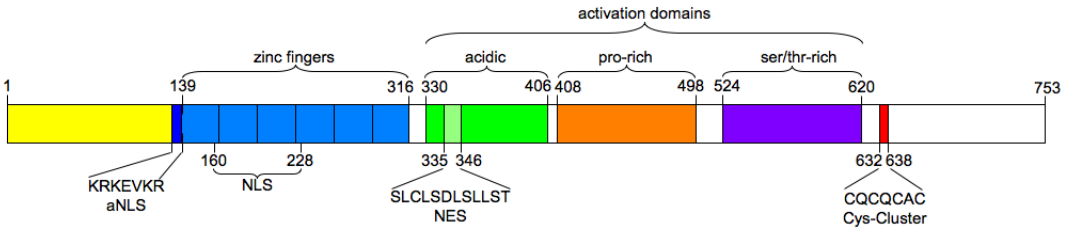

Figure 4: Functional domains of hMTF-1. Shown are the six zinc fingers, the three transcriptional activation domains, the nuclear localization and nuclear export signals and the C-terminal cysteine cluster.

The C-terminal cysteine cluster was previously shown to be important for metal-induced activity of human and mouse MTF-1 (15, 33). This cluster is conserved throughout the vertebrate MTF-1 homologs and it was proposed that it participates in the metal-sensing ability of MTF-1. In part I of this work we identify this cysteine cluster as a novel protein-protein interaction surface which mediates homodimerization of human MTF-1.

Interaction partners of human and mouse MTF-1

To date several proteins have been identified which interact or cooperate with MTF-1 at the DNA level. The transcription factor Sp1, for example, was shown to bind to the promoter region of the human MT-IIA gene (39, 61). Sp1 recognizes the CCGCCC core motif, and is a transcriptional activator of a large number of genes (92). In the case of the MT-IIA promoter, Sp1 acts as a repressor, possibly by interfering with MTF-1 for DNA binding. The role of NF-1 (nuclear factor-1) in metallothionein expression is controversial. On the one hand it was shown that NF-1, if overexpressed, inhibits mouse MT-1 reporter gene expression (35, 53). On the other

hand it was suggested recently that NF-1 boosts metal-dependent activation of the MT-1 gene by MTF-1 (43). Similarly, the transcription factor USF1 (upstream stimulatory factor-1) cooperates with MTF-1 to induce mouse MT-1 gene expression in response to cadmium and oxidative stress (4).

MTF-1 interacts with two hypoxia-sensitive transcription factors, NF-κB and HIF-1α. The expression of the placental growth factor (PlGF) is induced under proinflammatory stimuli and hypoxia and is dependent on MTF-1 (27). NF-κB directly interacts with MTF-1 under hypoxic conditions and the resulting complex activates the expression of PlGF (19). HIF-1α is a subunit of the hypoxia inducible factor-1 (HIF-1), which is the main regulator of gene expression in response to hypoxia (37). Both MTF-1 and HIF-1α are recruited to the MT-1 promoter in response to hypoxia and both factors are needed to drive metallothionein expression under these conditions (59).

It was also suggested that MTF-1 represses the heat shock response as it suppresses activation of the Hsp70 promoter when overexpressed in HeLa cells and binds to the heat shock factor-1 (HSF-1)/heat shock response element (HRE) complex in bandshift experiments (87).

The interaction of MTF-1 with the transcriptional coactivator p300/CBP and Sp1 was reported to be induced by zinc treatment, with conserved leucine residues in the acidic activation domain of MTF-1 were crucial for this interaction (45). p300 and the closely related CBP (CREB-binding protein) have histone acetyl transferase (HAT) activity and are critical regulators of transcription in cells of higher eukaryotes, including flies, worms and plants (26, 36). They interact with DNA-binding transcription factors and coactivate transcription of target genes in several ways. First, p300/CBP bridges transcription factors to the polymerase II holoenzyme; second, they change chromatin structure into a more accessible configuration for the transcription machinery by acetylating histones near to the target promoters. p300 and CBP can also acetylate transcription factors directly, and thereby change their activity (82). In part II of this work we show that human MTF-1, like mouse MTF-1, is a direct interaction partner of p300 and is acetylated by p300 *in vitro*.

Functional domains and regulatory mechanisms of *Drosophila* MTF-1

Drosophila and human MTF-1 share 39 % protein sequence similarity, whereas the zinc fingers are best conserved with 78 % similarity (97). While hMTF-1 shows a strong induction in response to zinc and cadmium, dMTF-1 is best activated by copper and cadmium. In contrast to the fast and strong activation of hMTF-1 by zinc, copper hardly induces hMTF-1 as judged by a 4xMREd-reporter plasmid and induction can only be observed by treatment which exceeds 8 hours (U. Lindert and W. Schaffner, unpublished).

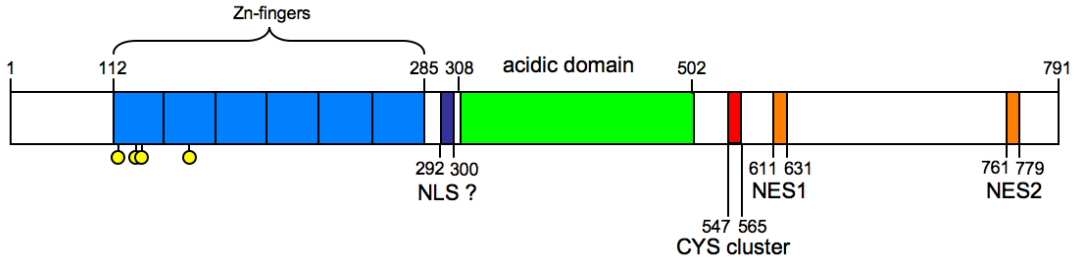

Figure 5: Domain structure of dMTF-1. The six zinc fingers mediate DNA binding to the MRE and the cysteine cluster was shown to be responsible for copper sensing. Putative functional domains are the acidic activation domain, the nuclear localization signal (NLS) and two nuclear export signals (NES). Phosphorylation sites identified by the Phosphopep project (11) are shown as yellow circles.

Like vertebrate MTF-1, the fly homolog has six C2H2-zinc fingers in the N-terminal part. Recently a proteomics approach, in which 4600 distinct phosphopeptides in the *Drosophila* cell line Kc167 were identified, revealed four phosphorylation sites located in zinc fingers 1 and 2 of dMTF-1, namely Tyr118, Ser126, Thr127 and Ser160 (11).

The domain structure of dMTF-1 has not been extensively studied so far, but an acidic activation domain located C-terminal of the zinc finger region was predicted according to the protein sequence. Furthermore, a putative nuclear localization signal, characterized by a short stretch of high lysine content, and two nuclear export signals, which usually contain 4-5 hydrophobic residues in a 10 amino acid stretch, were found by sequence inspection. A cysteine cluster (aa 547-565) is important for

sensing excess intracellular copper (14). Flies in which these cysteines are mutated fail to activate MtnA in response to high copper and show an impaired survival if grown on copper-containing food. dMTF-1 is also essential for survival under copper starvation as it activates the copper importer Ctr1B (79). This function is not impaired in the cysteine cluster mutant. Amino acids 657 – 791 display a high content of cysteines and this region was therefore denominated as a metallothionein-like domain (14). Interestingly, the C-terminal 40 amino acids of dMTF-1 are highly conserved among insect species, including mosquitoes and the honey bee *Apis mellifera.*

In part III of this thesis we describe an approach to further characterize the domain structure of dMTF-1, which includes identifying the activation domains of dMTF-1 by a one-hybrid approach, elucidating the importance of the identified phosphorylation sites, verifying the putative NLS and NES sequences and testing different truncation mutants for their transcriptional activity in cell culture and the fly model. These investigations should shed more light on the regulation of dMTF-1 and allow a comparison of *Drosophila* and human MTF-1. An interesting aspect would be to identify the regulatory domain that confers sensing of copper deficiency to dMTF-1.

References to Introduction

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A conserved cysteine cluster, essential for transcriptional activity, mediates homodimerization of human metal-responsive transcription factor-1 (MTF-1)

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Abstract

Metal-responsive transcription factor-1 (MTF-1) is an evolutionarily conserved zinc finger protein that activates transcription in response to heavy metals such as Zn(II), Cd(II) and Cu(I). The DNA-binding domain of MTF-1 recognizes a specific DNA sequence termed the metal response element (MRE), located in the promoter/enhancer region of its target genes. Here we show that human MTF-1 forms homodimers *in vivo* via a conserved cysteine-rich cluster "CQCQCAC" located near the C-terminus that was previously shown by cell transfection experiments to be important for basal and especially metal-induced transcriptional activity. Using a mammalian two-hybrid system we find that dimerization is not further enhanced by zinc treatment, implying that dimerization via the cysteine cluster is required for metal-induced transcriptional activity but actual zinc sensing is mediated by another domain. By contrast copper, which on its own activates hMTF-1 only weakly, stabilizes the dimer by inducing intermolecular disulfide bond formation via the cysteines and can synergize with zinc to boost hMTF-1 dependent transcription. A mutant protein in which these cysteines are replaced by alanines is still able to translocate to the nucleus in response to zinc treatment and bind to DNA in a cell free system. Therefore we propose that homodimerization is important to form an interaction platform for transcriptional coactivators.

Introduction

A typical transcription factor is composed of several functional domains that regulate its DNA-binding, transcriptional activity and subcellular distribution. Metal-responsive transcription factor-1 (MTF-1) is a transcription factor that is activated by heavy metals (1,2) and other stressors like hypoxia (3) and oxidative stress (4). Despite intense research the protein domains that regulate its activity in response to these stresses are still not all identified.

MTF-1's DNA-binding domain consists of six zinc fingers that mediate binding to the cognate DNA motif termed metal-response element (MRE), with the consensus sequence "TGCRCNC". Several copies of MREs are found in the promoter/enhancer region of MTF-1 target genes, of which the metallotionein genes are the best studied ones (4,5). Other target genes include *ZnT-1*, encoding a zinc efflux transporter (6), and the cadmium-responsive genes selenoprotein W, muscle 1 gene (*Sepw1*), N-myc downstream regulated gene 1 (*Ndrg1*) and cysteine- and glycine-rich protein 1 gene (*Csrp1*) (7). The fact that zinc-binding affinities can vary between zinc fingers (8,9) and that MTF-1 requires elevated levels of zinc in cell free DNA binding studies (5) implied that at least some of its zinc fingers participate in sensing cellular zinc levels. Studies on the role of individual zinc fingers in zinc sensing have yielded ambiguous results so far. On the one hand it was suggested that zinc fingers 5 and 6 can act as zinc sensors to mediate metal-responsive transcription, whereas zinc fingers 1 to 4 are representing the core DNA-binding domain (10). Other studies imply that also zinc finger 1 is involved in zinc sensing (11). Recent data also implicate the "linker" peptide between zinc finger 1 and 2 as being involved in zinc sensing (12).

Based on the presence of a characteristic amino acid composition (13), three transcriptional activation domains were ascribed to MTF-1 (14), each of them showing transcriptional activity when fused to a heterologous DNA-binding domain. The acidic activation domain confers the strongest transcriptional activity and is also able to mediate metal-induced transcription when fused to the DNA-binding domain of the yeast transcription factor Gal4, at least in a subset of cell lines that were tested (15). Additionally, kinase inhibitor studies implied that MTF-1 is regulated by phosphorylation through the action of protein kinase C, tyrosine kinases, casein kinase II and c-Jun N-terminal kinase (16,17).

Under standard conditions, MTF-1 mainly resides in the cytoplasm but rapidly translocates to the nucleus upon metal load (18,19). The subcellular localization of MTF-1 is regulated by a non-conventional nuclear localization signal (NLS), which extends over the first three zinc fingers. In addition, a cluster of basic amino acids located N-terminal to the zinc finger region contributes to nuclear import as an "auxiliary" NLS (15,18). A nuclear export signal overlapping with the acidic activation domain confers Crm1-dependent nuclear export to MTF-1. The functional relevance of export is unclear, as a constitutively nuclear protein is still able to mediate metal-responsive transcription (15).

A cluster of four cysteines ^{632}CQCQCAC^{638} (Figure 1A and B) close to the C-terminus of hMTF-1 is conserved in all vertebrate orthologs. It was previously shown that single and double substitutions of the cysteines in the cluster decrease the metal-induced transcriptional response (20,21). Such a domain, with the same spacing of cysteines was

this far not described for other transcription factors and the mechanism of its function remained unclear.

However, other metal-responsive transcription factors in lower eukaryotes contain regulatory cysteine clusters with different overall amino acid sequences that serve diverse functions. Cuf1 activates expression of copper import genes under copper deprivation in *S. pombe* (22). This transcription factor contains a cysteine rich region within its C-terminal region that, when bound to copper, blocks the nuclear localization signal by an intramolecular interaction thereby preventing nuclear accumulation and target gene transcription (23). In baker's yeast the metal-sensitive transcription factor Mac1p also responds to low copper availability but via a different mechanism (24,25). Its C-terminal region harbors two cysteine-rich regions. In a copper-bound state, they lead to an intramolecular interaction between the DNA-binding domain and the transactivation domain, thereby inhibiting both functions (26). Another transcription factor of *S. cerevisiae*, ACE,1 activates transcription of the CUP1 metallothionein gene in response to copper load (27). Here, copper binds to a cluster of several cysteines overlapping the DNA-binding domain and induces a conformational change that allows DNA-binding (28,29).

In the present study, we show that the C-terminal cysteine cluster of human MTF-1 (hMTF-1) mediates homodimerization and that metal-induced transcription depends on this dimerization. Nucleo-cytoplasmic shuttling and DNA-binding are not affected in the cysteine mutant that is unable to dimerize. Exposure to elevated concentrations of zinc, a condition that induces the transcriptional activity of hMTF-1, did not increase dimerization, demonstrating that even though dimerization via the cysteines is a

prerequisite for metal-induced transcription, it is not participating in the process of zinc-sensing. However copper, which on its own activates hMTF-1 poorly in the cell lines tested, stabilizes the dimer through oxidation of cysteines and thereby synergizes with zinc to boost transcription.

Materials and Methods

Cell culture and transfection

HEK293, HEK293T, U2OS, HeLa and mouse MTF-1 -/- cells (dko7 (5,14)), were cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) containing 8 % fetal bovine serum (Biochrom AG), 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Invitrogen). For transfections with the calcium-phosphate method 20 µg of total amount of DNA were added per 10 cm dish and cells were washed 14 to 16 hours after transfections. Unless mentioned otherwise, metal treatments were done 40 hours after transfection for four hours.

Plasmid constructions

The expression vector containing a VSV-tagged hMTF-1 cDNA clone (aa1-743) under the control of the CMV promoter (hMTF-1-VSV) was described previously (18). The expression vector in which cysteines at positions 632, 634, 636 and 638 were replaced by alanines (CYSmut-VSV) and the vectors containing single substitutions were

generated based on hMTF-1-VSV using site directed mutagenesis (QuikChange, Stratagene) according to the manufacturer´s instructions. The expression plasmids containing FLAG-tagged hMTF-1 truncations (FLAG-1-321, FLAG-1-511) were produced by cloning corresponding PCR fragments into the EcoRV/Xho1 sites of pCATCH-FLAG (30). FLAG-322-end, 322-endCYSmut-VSV, 322-end-VSV, FLAG-hMTF-1 and FLAG-CYSmut were generated using PCR technology and restriction enzyme digestion/ligation based on the described plasmids. The reference and reporter plasmids CMV-OVEC-Ref, OVEC-Ref (SV40 promoter) (31), 5xGOVEC (containing five Gal4 binding sites in its promoter region) (32), 4xMREd-OVEC (31), mMT1-OVEC (2) and hMT2a-OVEC (33) were described previously. 2xGOVEC, 4xGOVEC and 8xMREd-OVEC were produced by fusing corresponding annealed oligonucleotides into the OVEC-vector (31). Gal4-DBD fusion constructs are based on the vector pSCTGal(1-93) containing the DNA-binding domain, amino acids 1-93 of the yeast transcription factor Gal4 (13). To generate VP16-fusion constructs the acidic transcriptional activation domain of the viral VP16 protein (amino acids 413-490 (13)) was fused to various hMTF-1 clones. Sequences of used oligonucleotides and detailed cloning strategies are available on request.

The p300-FLAG expression plasmid was a generous gift of Dr. Michael Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, University of Zürich).

Co-immunoprecipitations and immunoblotting

Cell lysates, originating from a confluent 10 cm dish of HEK293T cells transfected with 2 µg of expression clone, were prepared in a lysis buffer containing 50 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 % NP-40, 1 mM DTT and protease inhibitors. The cleared lysates were incubated with 1 µl anti-VSV antibody (Sigma, V5507) for 1 h at 4 °C, followed by 1 h of incubation with 20 µl of Protein-G Sepharose Fast Flow (GE Healthcare). The sepharose beads were washed four times with lysis buffer and precipitates were eluted by boiling 5 minutes in Laemmli buffer containing 2.5 % β-mercaptoethanol. Proteins were separated by 7.5 or 10 % tris-glycine SDS-PAGE (BIO-RAD) and transferred on a PVDF-membrane (Amersham). VSV- and FLAG-tagged proteins were detected using 1:10,000 and 1:1000 dilutions of anti-VSV and anti-FLAG (Sigma, F1804) antibody, respectively, followed by a horse radish peroxidase-conjugate anti-mouse IgG at 1:10,000 dilution (GE Healthcare, NA931). Proteins were visualized using the ECL chemiluminescent detection system (Pierce).

S1 nuclease protection assay

Cells of an exponentially growing 10 cm culture dish were transfected with 0.05-2.5 µg of the particular expression clones, 10 µg of reporter and 1-5 µg of reference plasmid (CMV-OVEC-Ref or OVEC-Ref depending on the cell line), as indicated in the figure legends. Herring sperm DNA was added as a carrier to each sample to a total of 20 µg. Isolation of RNA and the S1 nuclease protection assay were done as described previously (31,34). Signals were visualized using the fluorescent image analyzer FLA-

7000 and quantified using the ImageGauge software (Fujifilm life science). Reporter signals were normalized to the reference signals.

Preparation of nuclear extracts and electric mobility shift assay (EMSA)

Preparation of nuclear extracts from transiently transfected HEK293T or dko7 cells was performed according to (35). Binding reactions and gel conditions were described in (2). 13 µg of nuclear extract and 40 fmol of end-labeled MRE-s oligonucleotide for Figure 3C and 30 µg of nuclear extract and 600 pmol of end-labeled MRE-s oligonucleotide for Figure 5D were used. Binding reactions where cold competitors in 50-fold excess were used are indicated in the figure legends.

Chemical crosslinking

Cell lysates were prepared using a buffer containing 20 mM HEPES pH 8, 150 mM NaCl, 1 % Triton X-100, 5 mM DTT and protease inhibitors. Protein concentrations were measured with the Bio-Rad protein assay (Bio-Rad,); 3.7 µg/µl of protein were incubated with 5 mM EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) for 30 minutes at room temperature in the dark. The crosslinking reaction was stopped by adding Laemmli sample buffer containing 2.5 % of beta-mercaptoethanol and samples were analysed using SDS-PAGE.

Indirect immunofluorescence

Exponentially growing cells of a 10 cm dish were transfected with 1 or 5 µg (for HEK293T or U2OS cells, respectively) of the indicated expression plasmid and distributed on coverslips the next day. Cells were either left untreated, or treated with 100 µM $ZnSO_4$ for 3 hours. The immunofluorescence staining was performed as described previously (18) using a mouse anti-VSV (Sigma, V5507) and an Alexa Fluor 546 goat anti-mouse antibody (Invitrogen, A11030). Nuclei were stained by 4′,6-Diamidino-2-phenylindol (DAPI) at a concentration of 1 µg/ml for 1 minute.

Results

Mutation of the cysteine cluster hampers transcriptional activity

To investigate the role of the conserved C-terminal cysteine cluster of hMTF-1 we replaced all four cysteines by alanines, here after referred to as cysteine mutant ("CYSmut" in figure legends), and also tested single substitutions. The transcriptional activity was analyzed in a mouse cell line lacking MTF-1 (dko7) with MRE-containing reporter constructs. Reporter and reference transcript levels were quantified using a S1-nuclease protection assay. The wild type protein induced expression of a reporter containing four copies of MREs (4xMREd-OVEC) 6- and 8-fold in response to 100 µM zinc and 50 µM cadmium, respectively (Figure 2A). Mutation of all four cysteines almost completely abolished zinc- and cadmium-induced transcription. The basal activity was reduced to 50 % of wild type level, indicating that the cysteines, besides

their major role in mediating strong metal-induced transcription (20), also contribute to basal transcriptional activity. Single substitutions decreased activity only slightly. The fact that the response to cadmium is strongly affected in the case of the C634A mutant, but was almost unchanged if Cys638 is mutated, might indicate a metal specificity which remains to be further investigated. The cysteine mutant also failed to drive transcription from natural promoters, namely, of mouse *MT1* and human *MT2a*, as shown in Figure 2C. The failure to induce adequate transcription was not due to a reduced stability of the mutant protein, because the level of expression was the same for the wild type and cysteine cluster mutant protein (Figure 5A).

Nuclear translocation upon metal treatment and DNA binding is not affected in the cysteine mutant

A hallmark in the activation of MTF-1 is its rapid nuclear translocation upon exposure to heavy metals and other stress conditions (18,19). To investigate if the cysteine mutant does not activate gene expression because this translocation step is hampered, VSV-tagged cDNA-clones were transfected into U2OS and HEK293T cells and the localization of the proteins monitored. Quantitative analysis in U2OS cells revealed that cytoplasmic-nuclear translocation is the same for wild type and the cysteine mutant protein (Figure 3A). Likewise, nuclear translocation upon metal treatment is observed in HEK293T cells for wild type and mutant protein (Figure 3B).

Further, to determine if the four cysteines are needed for proper DNA binding, we performed a bandshift assay with a radiolabeled oligonucleotide containing the

consensus MRE sequence (MRE-s) and nuclear extracts isolated from MTF-1-/- fibroblasts (dko7) transfected with a cDNA expression plasmid encoding either the wild type or mutant MTF-1 protein. As previously reported, pretreatment of the cells with zinc for four hours increases the bandshift signal, due to increased nuclear localization of MTF-1 (33). Mutation of four cysteines of the cluster did not prevent binding of MTF-1 to DNA in this *in vitro* assay (Figure 3C). Altogether these data show that the inability of the mutant protein to activate transcription is not due to a failure in DNA binding or nuclear translocation.

hMTF-1 homodimer formation depends on the cysteine cluster

Inspired by the studies with the yeast transcription factors Mac1p and Cuf1 we tested whether the cysteine cluster of hMTF-1 drives an essential intramolecular interaction with other regulatory regions, such as the zinc fingers, the NLS and NES sequences and the acidic- and proline-rich activation domain. To this end we performed co-immunoprecipitation on lysates of transiently transfected HEK293T cells with differentially tagged versions of full length MTF-1 and/or subsegments thereof. A strong interaction was observed between full length proteins (Fig. 4A), but no interaction could be detected between the N-terminal (aa1-511 or aa1-321) and the C-terminal domain (aa322-end) (Figure 4C). Interestingly, the C-terminal half, missing the zinc finger region, was sufficient to mediate a self interaction (Fig. 4B), which indicates that the interaction can occur independently of DNA-binding.

We next tested if mutation of the cysteines abolishes the observed self-association. As shown in Figure 5A the interaction as measured by the amount that co-immunoprecipitated, is almost completely abrogated for the cysteine mutant protein. The fact that interaction is equally lost if one or both proteins are mutated in this assay suggests that dimerization occurs between the cysteine clusters of both proteins rather than between a cysteine cluster on one partner and a different domain in the other. To determine the stoichiometry of the hMTF-1 complex we performed a crosslinking reaction with the zero length cross-linker EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride), which produces isopeptide bonds between carboxyl groups and primary amines. Cell lysates of HEK293T cells transiently transfected with the C-terminal construct (aa322-end) were treated with 5 mM EDC for 30 minutes. Comparison with the molecular weight standards revealed that the slower migrating band in EDC treated cell lysates corresponds to a dimer (Figure 5B). The dimer band was not observed for the cysteine mutant protein. The amount of crosslinked dimer in our assay is relatively low, presumably because the optimal pH for crosslinking with EDC is at a non-physiological pH between 4.7 and 6.0, while the crosslinking reaction showed here was carried out at pH 7.9, as no crosslinking of MTF-1 and no co-immunoprecipitation of hMTF-1 dimers was observed at pH 7.0 or lower (not shown). Therefore we cannot determine from this data whether only part or all of MTF-1 is present in dimeric form *in vivo.*

We wondered why in a standard bandshift assay there was no evidence for MTF-1 dimerization resulting in a supershift, and considered the possibility that MTF-1 was too dilute and/or the conditions during gel electrophoresis are unfavorable to maintain the dimer. Indeed, with higher amounts of nuclear extract from HEK293T cells transfected

with a hMTF-1 expression vector and higher concentrated labeled probe in the binding reaction, we observed a slower migrating band which was not present with the mutant protein (Figure 5D). If the C-terminal half was coexpressed with the full length protein, an intermediate size band was observed, indicative a heterodimer forming between a full length MTF-1 and the C-terminal fragment. This intermediate band was absent if the four cysteines of the C-terminal cluster were mutated, showing that the protein-protein interaction is dependent on the cysteine cluster.

To see if a metal ion, especially zinc, is part of the dimerization reaction, we performed a co-immunoprecipitation in the presence of the zinc chelators 1,10-orthophenanthroline or TPEN. This experiment was performed with the C-terminal construct, since adding chelators might remove zinc from the N-terminal zinc fingers and lead to misfolding and aggregation of the proteins. None of the chelators eliminated the dimerization reaction of the C-terminal hMTF-1 construct (Figure 5C), which might mean that the dimers are not involving zinc coordination or that metal ions are tightly packed in between the cysteines and thus inaccessible to the chelators.

hMTF-1 homodimerization is not increased by zinc treatment

To assess if the extent of dimerization is responsive to metal treatment and thus might be an important component of metal sensing by MTF-1, we also pretreated the cells for four hours with zinc prior to cell lysis and co-immunoprecipitation. This did not change the apparent strength of the interaction, even if additional zinc was added to the lysis buffer (data not shown).

A similar result was observed in a mammalian two-hybrid assay performed in U2OS cells. A C-terminal segment (aa 515-end) of hMTF-1 was fused to the DNA-binding domain of the yeast transcription factor Gal4 (aa1-93) or the activation domain of the herpes simplex virus transcription factor VP16. Only the serine/threonine-rich activation domain of hMTF-1 is present in these constructs, and the resulting Gal4 fusion protein shows a weak transcriptional activity on its own (see Figure 7A). When the two constructs, Gal4-515-end-VSV and 515-end-VP16, were coexpressed we observed a robust reporter gene expression. The interaction and the resultant reporter gene expression were severely reduced by mutation of the four cysteines in one or both partners (Figure 6). Single substitutions had less effect, resulting in only a slightly reduced two-hybrid signal (data not shown). Pretreatment of the cells with zinc for 4 hours, a treatment increasing MTF-1's transcriptional activity, did not boost the transactivation of the reporter gene, supporting the notion that the cysteines are not used for zinc sensing (Figure 7A). Even prolonged treatment with zinc had no effect on the intensity of the two-hybrid interaction. Dko7 cells, which lack MTF-1, could not be used in this assay since the activity of the viral VP16 activation domain alone was elevated by treating these cells with zinc.

Copper pretreatment induces dimerization via formation of intermolecular disulfide bonds and cooperates with zinc for transcription.

As mentioned before, zinc treatment did not enhance the interaction between the cysteine clusters. We also tested cadmium and copper in a time course experiment to

investigate if these metals increase the homodimerization of hMTF-1. Copper and cadmium strongly boosted the two-hybrid interaction (Figure 7A). We also tested menadione, a drug that, like copper, undergoes redox cycling and induces oxidative stress. However, menadione failed to induce the dimerization indicating that this effect is restricted to metal-induced oxidative stress. In further studies we concentrated on the effect of copper which on its own, in contrast to cadmium, barely induces MTF-1 activity in the cell lines tested. When lysates of HEK293T cells overexpressing the C-terminal construct were resolved by PAGE under non-reducing gel conditions, a higher molecular weight band was observed in the case of copper- but not zinc-treated cells (Figure 7B). The band was destroyed by addition of the reducing agent β-mercaptoethanol. This indicates that in the case of copper, the dimerization is likely reinforced by the formation of disulfide bonds. To test if this covalent link is beneficial or detrimental for MTF-1 function, the zinc-induced activity of endogenous MTF-1 was measured on a 4xMREd-reporter in U2OS cells that were pretreated with different concentrations of copper for 16 hours. As seen in Figure 7C, prolonged exposure to copper, which on its own is a very poor inducer of MTF-1 activity, enhances the increase in activity of MTF-1 following zinc treatment.

MTF-1 activity can be partially rescued by a heterologous dimerization domain

In a one-hybrid experiment, for which the C-terminal domain of MTF-1 covering all transactivation domains and the cysteine cluster (aa322-end) was fused to the Gal4 DNA-binding domain, the respective cysteine mutant reached, depending on the

number of Gal4 binding sites in the promoter region of the reporter plasmid, up to 75 % of the corresponding wild type protein activity (Figure 8). We consider it likely that homodimerization mediated via the Gal4 DNA-binding domain (36) can compensate for the effect of the cysteine mutant. In contrast, even on a promoter with eight tandem MREs the activity of the cysteine mutant is significantly impaired.

The cysteine cluster mutant can still bind to p300

It was previously found that p300 interacts with mouse MTF-1 and that this interaction is enhanced by treating the cells with zinc (37). The protein p300 is a ubiquitous coactivator protein of higher eukaryotes with histone acetyltransferase activity. We therefore tested if the cysteine mutant of human MTF-1 fails to activate transcription, because its interaction with p300 was impaired. To do this, in HEK293 cells, we transiently expressed tagged versions of p300 together with either wild type hMTF-1 or the cysteine cluster mutant. We found that MTF-1 and p300 co-immunoprecipitated and that this interaction was independent of the presence or absence of the cysteine cluster (Figure 9).

Discussion

The members of several families of eukaryotic transcription factors, like the bHLH, bZIP and the NF-kappaB transcription factors, need to dimerize to bind to regulatory

sequences in their target genes (38). Depending on the status of the cell, homodimers or heterodimers, typically within the same family of transcription factors, are formed that will activate a specific set of genes. Dimerization is not considered a common feature of C2H2 zinc finger transcription factors. In contrast to other DNA-binding domains the diversity of DNA binding specificities of C2H2 zinc fingers can be achieved by duplication and/or modifications of C2H2 motifs. Dimerization was nevertheless reported for a number of zinc finger transcription factors. The transcriptional regulator Ikaros, which contains four N-terminal zinc fingers that mediate DNA-binding, is homo- and heterodimerizing with other Ikaros family members via another two zinc fingers located in the C-terminus (39-41). Furthermore, a subfamily of C2H2 zinc finger proteins contains a highly conserved 84-residue motif, called the SCAN domain that mediates self-association as well as the selective association with other proteins (42,43).

Here we show that hMTF-1 homodimerizes via a novel protein interaction domain, characterized by a cluster of four cysteines of specific spacing located near the C-terminus of the protein. This cluster is conserved throughout the vertebrate homologs of MTF-1, which argues for its functional importance. Transcriptional activity, whether tested on natural metallothionein promoters or synthetic MRE-containing promoters, was shown to be dependent on the ability of hMTF-1 to dimerize. Cell free binding studies indicated that like the wild type protein the cysteine mutant of hMTF-1 can readily bind to DNA as a monomer. Using a one-hybrid approach we could show that the effect of the cysteine mutant can be largely rescued by addition of the homodimerizing Gal4 DNA-binding domain. That the cysteine cluster can be functionally replaced by a heterologous domain mediating homodimerization suggests that it only serves this function and that neighboring domains, such as the acidic

activation domain, depend on being in close proximity to fulfill their function. Bringing single MREs in close proximity, like in the 8xMREd-OVEC reporter plasmid in which the MRE-motifs are separated by an 11 bp spacer, is not sufficient to rescue the cysteine cluster mutant.

Zinc treatment did not increase MTF-1's dimerization as judged by the mammalian two-hybrid assay and co-immunprecipitation results. Additionally, the interaction could not be destroyed by adding metal chelators. These data, together with the fact that also basal transcriptional activity is reduced in the cysteine cluster mutant of hMTF-1, suggest that dimerization is not a step in the zinc-sensing process of hMTF-1. Accordingly, the zinc-sensing mechanism is likely restricted to the zinc fingers and the acidic activation domain, as shown previously (10,11,15).

In the cell lines used hMTF-1 is poorly responsive to elevated concentrations of copper. Therefore it was a surprising finding, that copper, as well as cadmium, induce dimerization measured by a two-hybrid assay. Non-reducing gel electrophoresis showed that copper is able to oxidize the cysteines of the cluster so that a covalently linked dimer is formed. This effect was most readily observed after a prolonged treatment, perhaps indicating an exhaustion of cellular antioxidant protection.

So far it is not clear how dimeric DNA-binding of hMTF-1 occurs. It is noteworthy that no conserved spacing of direct or inverted repeats of MREs has been found in the promoter and enhancer regions of MTF-1 target genes. The possibility that dimerization is needed to recruit distant regulatory DNA sequences via looping out of the intervening DNA seems unlikely, since a synthetic promoter with closely spaced, proximally

located MREs is also not activated by the cysteine cluster mutant. Additionally, changing MRE orientation in a synthetic promoter has no effect on reporter gene expression (U. Lindert and W.S., unpublished).

Taken together these data indicate that dimerization is most likely important to recruit factors of the transcriptional machinery, such as specific coactivator/mediator components. The transcriptional coactivator p300 was shown to interact directly with mouse MTF-1 in a zinc dependent manner (37) and hMTF-1 is acetylated by p300 *in vitro* (V.G. and W.S., unpublished). The cysteine cluster mutant can still bind to p300, but further studies are needed to define the interactome of wild type and mutant hMTF-1. In *Drosophila* it was shown that several components of the TFIID and Mediator coactivator complexes are recruited to the metallothionein A promoter in response to copper (44). It remains to be seen whether the mammalian homologs of these proteins are involved in a dimerization-dependent interaction with hMTF-1.

Drosophila and mammalian MTF-1 are highly similar in their zinc finger domain while the C-terminal part, containing the activation domains is less, if at all, conserved (45). Interestingly, despite the lack of similarity in the C-terminal part, *Drosophila* MTF-1 also contains a cysteine-rich cluster with a different spacing of cysteines ("CNCTNCKCDQTKSCHGGDC") that is conserved among all drosophilid species. This cluster was shown to be necessary for copper-induced metallothionein A gene transcription, a prerequisite for protecting the animals against copper intoxication. A different function of *Drosophila* MTF-1, namely, transcription of a copper importer gene in response to copper deprivation is not affected by mutation of this cysteine cluster (46), thus it is not generally required for transcriptional activity. The *Drosophila*

cysteine cluster was shown to bind copper and accordingly is expected to sense high copper levels in the cytoplasm. We suggest that the cysteine clusters of *Drosophila* and human MTF-1 evolved independently and serve different functions. Consistent with this notion the mammalian and the *Drosophila* cysteine clusters are not functionally interchangeable (20).

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48. Muylle F., R. J., De Coen W., Blust R. (2004) Cloning of alternatively spliced MTF-1 isoforms from rainbow trout, Oncorhynchus mykiss.

Legends to Figures

Figure 1:

Location and sequence alignment of the cysteine cluster. (A) Functional domains of human MTF-1, showing the six zinc fingers, the acidic, proline-rich and serine/threonine-rich activation domains, the nuclear localization signal (NLS) spanning zinc fingers 1 – 3, a basic stretch of amino acids nearby with auxiliary NLS function (aNLS), and the nuclear export signal (NES), overlapping with the acidic activation domain. The conserved cysteine cluster is located in the C-terminal part of the protein. (B) Protein sequence alignment of the region containing the cysteine cluster, showing the sequences of *Homo sapiens* (human), *Mus musculus* (mouse), *Bos taurus* (cow), *Gallus gallus* (chicken), *Xenopus laevis* (African clawed frog), *Takifugu rubripes* (Japanese pufferfish), *Cyprinus carpio* (common carp) and *Oncorhynchus mykiss* (rainbow trout). Several splice variants of MTF-1 were found for the common carp and the rainbow trout, among them only the longest versions contain the cysteine cluster (Uniprot database search (47,48)). For the zebrafish *Danio rerio* no splice variant containing the cysteine cluster was described so far, however a variant containing the cysteine cluster is possible considering splice site predictions (Kurt Steiner, W.S., unpublished).

Figure 2:

The cysteine cluster mutant fails to activate transcription from MRE-containing promoters. Dko7 cells were transfected with 10 µg of 4xMREd-OVEC (A), mMT1-OVEC or hMT2a-OVEC (B), 5 µg of OVEC-ref and 2 µg of the indicated wild type or mutant hMTF-1 expression vectors. Cells were treated with the indicated metals for four hours. Transcript levels were determined by the S1-nuclease protection assay and reporter signals were normalized to reference signals. The signal of untreated, with pC-hMTF-1-VSV transfected cells was set to 1. Error bars in (A) indicate the standard deviation of three independent experiments.

Figure 3:

Nuclear localization upon zinc treatment and DNA-binding is not disturbed by mutation of the cysteine cluster. The subcellular localization of hMTF-1-VSV or CYSmut-VSV was analyzed by indirect immunofluorescence in (A) U2OS and (B) HEK293T cells that were either left untreated (n.t.) or treated with 100 µM $ZnSO_4$ for 3 hours. (A) For each condition 200 cells were counted and classified according to the following categories. N: nuclear localization only; C<N: mostly nuclear localization; C=N: equal distribution; C>N: mostly cytoplasmic localization. (B) Immunofluorescent staining of transiently transfected HEK293T cells showing that the cysteine cluster mutant retains the ability to translocate to the nucleus upon zinc treatment. (C) Electrophoretic mobility shift assay of a P^{32}-labeled consensus MRE oligonucleotide (MRE-s) with nuclear extracts of dko7 cells that were not transfected (n.t.) or transiently

transfected with either wild type or mutant hMTF-1. Cells were treated with 100 µM $ZnSO_4$ prior to cell harvest if indicated. Specific (MRE-s) and unspecific (Sp1 binding site) unlabeled competitor oligonucleotides were added in lines 4 and 5, respectively. An unspecific band "u.b." might be caused by the buffer front.

Figure 4:

hMTF-1 self-associates via its C-terminal half. (A) HEK293T cells were transfected with 2 µg of VSV- and FLAG-tagged hMTF-1 expression plasmids, followed by co-immunoprecipitations with an anti-VSV antibody. Immunoblots were developed with either anti-FLAG or anti-VSV antibodies. (B) Co-immunoprecipitation of FLAG-322-end and 322-end-VSV demonstrate that the C-terminal half of the protein is sufficient for dimerization. The failure of FLAG-1-322 or FLAG-1-515 to co-immunoprecipitate with 322-end-VSV constructs is shown in (C). WB: Western blot; IP: immunoprecipitation; α: antibody.

Figure 5:

The dimerization of hMTF-1 is dependent on the cysteine cluster. (A) HEK293T cells were transfected with the indicated plasmids and whole cell lysates were analyzed by co-immunoprecipitations with anti-VSV antibodies. (B) To determine the stoichiometry of the hMTF-1 complex whole cell extracts of HEK293T cells expressing

322-end-VSV were chemically crosslinked with EDC. The molecular weight marker is indicated at the left of the figure. (C) Co-immunoprecipitations were performed with whole cell extracts of HEK293T cells transfected with an expression vector containing 322-end-VSV in presence of 0.5 or 1 mM 1,10-orthophenanthroline (Phen) or 0.1 or 0.2 mM TPEN revealing that the interaction persists in the presence of these metal chelators. The chelators were not added to live cells prior to lysate preparation, as they induce a rapid detachment of the cells followed by apoptosis. WB: Western blot; IP: immunoprecipitation; α: antibody. (D) Bandshift analysis using a P^{32}-labeled MRE-s oligonucleotide and nuclear extracts of HEK293T cells that were transfected with the indicated plasmids. On the right-hand side, the predicted DNA-protein complexes that would explain the bands are shown schematically.

Figure 6:

MTF-1 dimerization is also demonstrated by a mammalian two-hybrid system. To measure the two-hybrid interaction in HeLa cells (black bars) 10 µg of 5xGOVEC reporter plasmid, 3 µg of OVEC-Ref and 2 µg of Gal4- and VP16-fusion constructs were transfected as indicated. U2OS cells (grey bars) were transfected with 10 µg of 5xGOVEC reporter plasmid, 1 µg of CMV-OVEC-Ref and 2 µg of Gal4- and VP16-fusion constructs. RNA was isolated and expression levels of reporter and reference genes were analyzed by the S1-nuclease protection assay. Reporter gene transcript levels were normalized to reference gene transcript levels and compared to the sample value of Gal-515-end-VSV/515-end-VP16, which was set to 1.

Figure 7:

Copper and cadmium, but not zinc, induce dimerization of hMTF-1. Copper pretreatment synergizes with zinc induction of hMTF-1 transcriptional activity. (A) To test if metals have an effect on MTF-1 dimerization in the two-hybrid assay, U2OS cells were transfected with 10 µg of 5xGOVEC, 1 µg of CMV-OVEC-Ref and 2 µg of Gal-515-end-VSV and 515-end-VP16 expression plasmids. Prior to cell harvest the cells were treated with $ZnSO_4$, $CdCl_2$, $CuSO_4$ or menadione for the indicated times. Normalized values were compared to the sample value of non-treated cells (n.t.), which was set to 1. The activity of the "bait" construct Gal-515-end-VSV alone is marked by an asterisk. Error bars indicate standard deviations of three independent experiments. (B) HEK293T cells were transfected with 322-end-VSV expression plasmid and treated with the indicated metals for 16 hours. Cleared cell lysates were separated by non-reducing (lanes 1-3) and reducing (lanes 4-6) SDS-PAGE and Western blots were developed with an anti-VSV antibody. Copper, but not zinc, induces an intermolecular disulfide bond formation. (C) To analyze if copper pretreatment affects zinc-induced transcription via hMTF-1, U2OS cells were transfected with 10 µg 4xMREd-OVEC and 1 µg of CMV-OVEC-Ref and treated with different $CuSO_4$ concentrations 12 hrs before $ZnSO_4$ addition. The normalized value of untreated cells was set to 1.

Figure 8:

The cysteine cluster mutant can be largely rescued by the Gal4 DNA-binding domain that forms homodimers. Dko7 cells were transfected with 10 µg of the

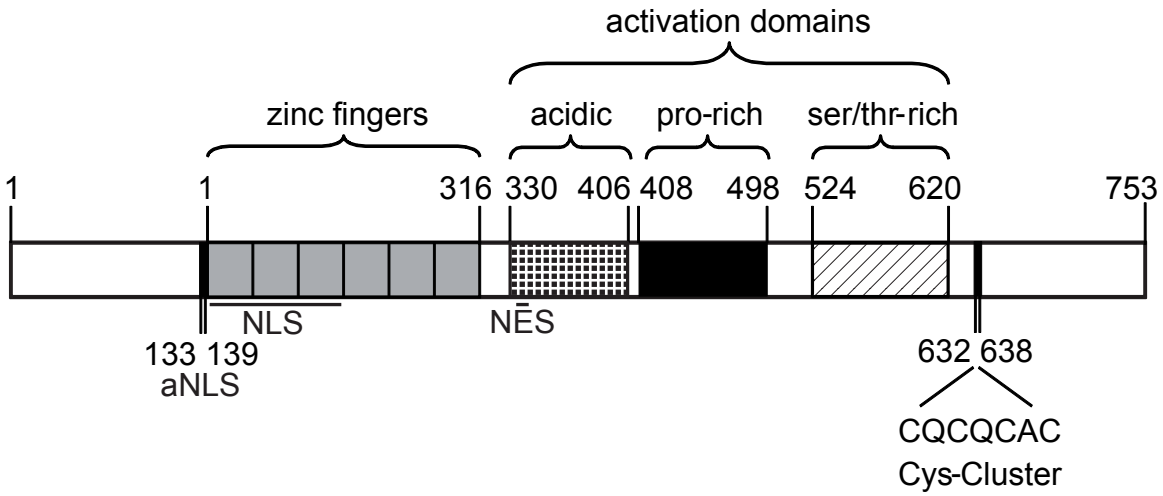
corresponding reporter construct and 3 µg OVEC-Ref, together with 2 µg and 0.05 µg of hMTF-1 and Gal4-fusion clone, respectively. Transcripts were measured by the S1-nuclease protection assay. Reporter signals were normalized to the reference signal and compared to the sample value of uninduced cells transfected with full length hMTF-1, which was set to 1. Error bars indicate standard deviations of three independent experiments.

Figure 9:

The cysteine cluster mutant can interact with the transcriptional coactivator p300. HEK293 cells that transiently express hMTF-1-VSV or CYSmut-VSV together with FLAG-p300 were treated for 4 hours with 100 µM $ZnSO_4$ if indicated. Whole-cell extracts were immunoprecipitated (IP) using an anti-FLAG antibody. An interaction that is however independent of zinc supplementation is revealed by immunoprecipitates which were analyzed by Western blotting (WB), using an antibody against the VSV- or FLAG-epitope.

Figure 1

A



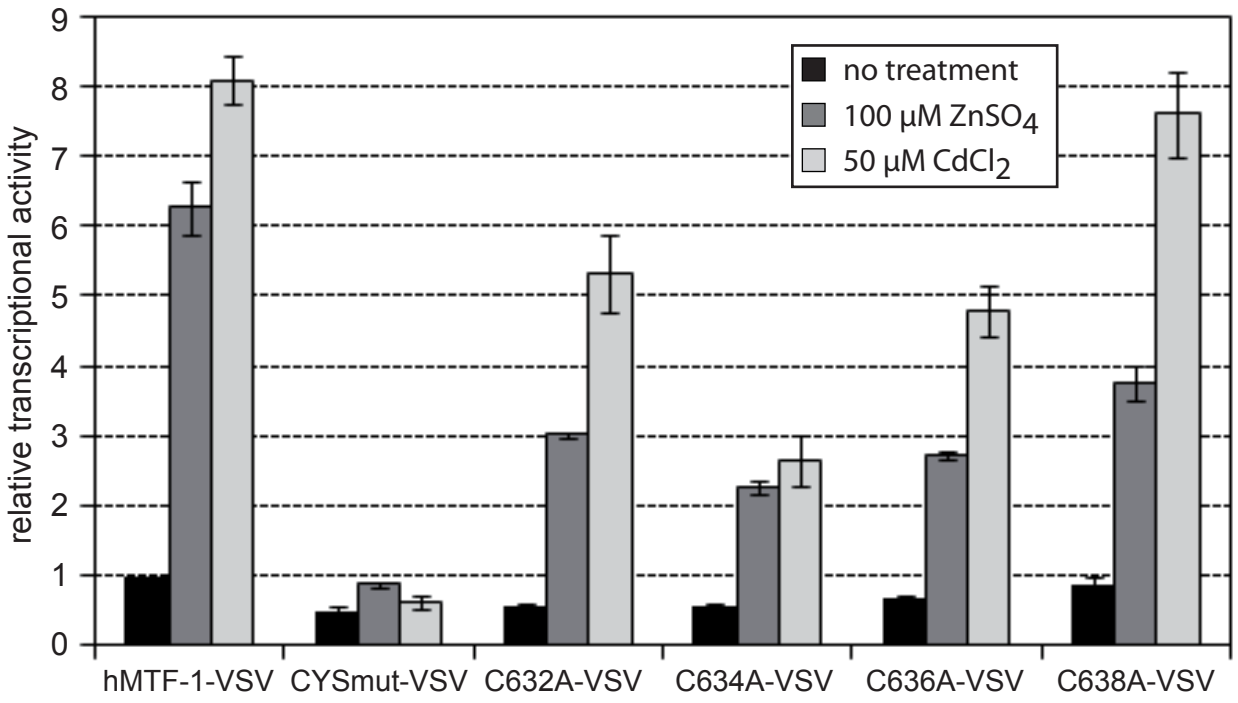
B

Cys-cluster

```
human          SSVQQIGLSVPVIIIKQEEACQCQCACRDSAKER--ASSRRKGCSSPP
mouse          SSVQQIGLSVPVIIIKQEEACQCQCACRDSAKER--AAGRRKGCSSPP
cow            SAVQQIGLSVPVIIIKQEEACQCQCACRDSAKER--AASRRKGCPSPP
chicken        NPVQQIGLSVPVIIIKQEEACQCQCACRDSAKDK--VTVKKESSSPEP
xenopus        NPVQQIGLSLPVLIIKQEESCQCQCACRDSAKDT--AN-KRKGCSPAP
takifugu       NSVQQIGLSLPVIIIKQEESCQCQCACRDSAKEK--NSKSSSSSMSAQ
common carp    NAVQQIGLSLPVIIIKQEESCQCQCACRDSGKDKSTASSVEKTKMSAQ
rainbow trout  TALQQLGLRLPVIVIRQGESCQCRCPCRDGSTAS--DTEKQTGCQPTG
               ..:**:** :**::*:* *:***:*.***...
```

Figure 2

A


relative transcriptional activity
0
1
2
3
4
5
6
7
8
9
no treatment
100 µM ZnSO4
50 µM CdCl2
hMTF-1-VSV
CYSmut-VSV
C632A-VSV
C634A-VSV
C636A-VSV
C638A-VSV

B

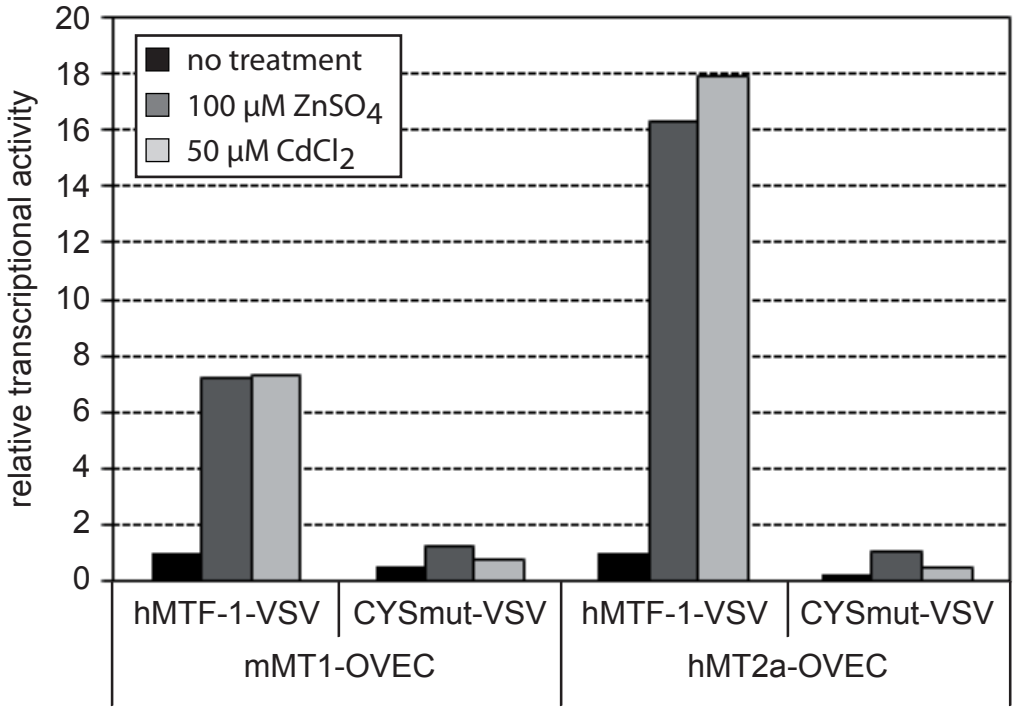
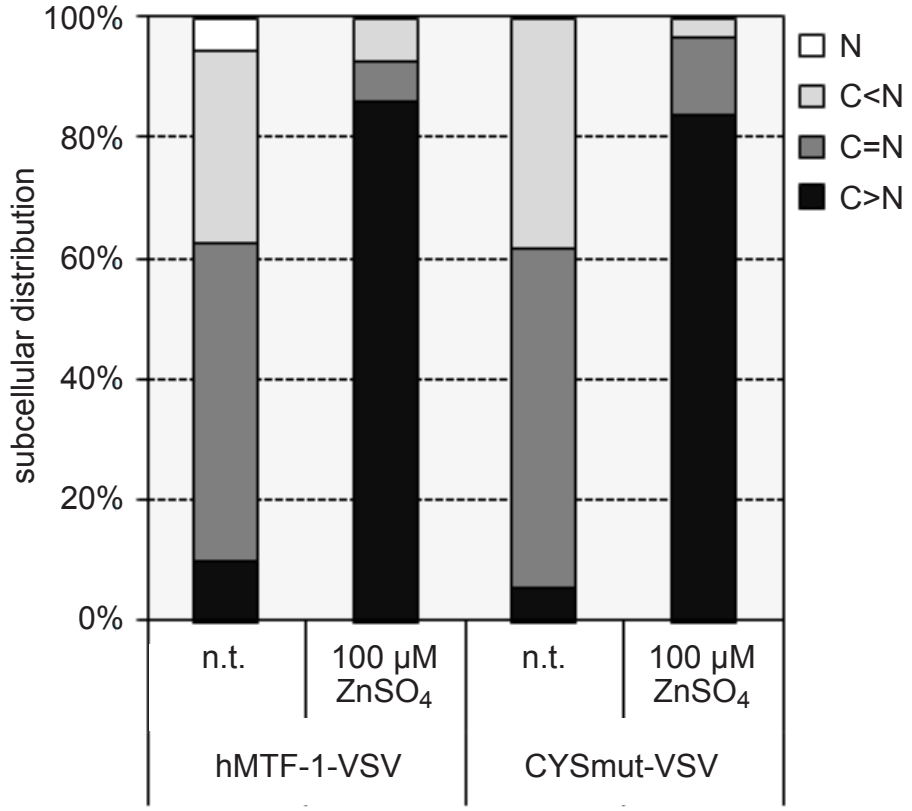

relative transcriptional activity
0
2
4
6
8
10
12
14
16
18
20
no treatment
100 µM ZnSO4
50 µM CdCl2
hMTF-1-VSV
CYSmut-VSV
hMTF-1-VSV
CYSmut-VSV
mMT1-OVEC
hMT2a-OVEC

Figure 3

A


100%
80%
60%
40%
20%
0%
subcellular distribution
N
C<N
C=N
C>N
n.t.
100 µM ZnSO4
n.t.
100 µM ZnSO4
hMTF-1-VSV
CYSmut-VSV

B

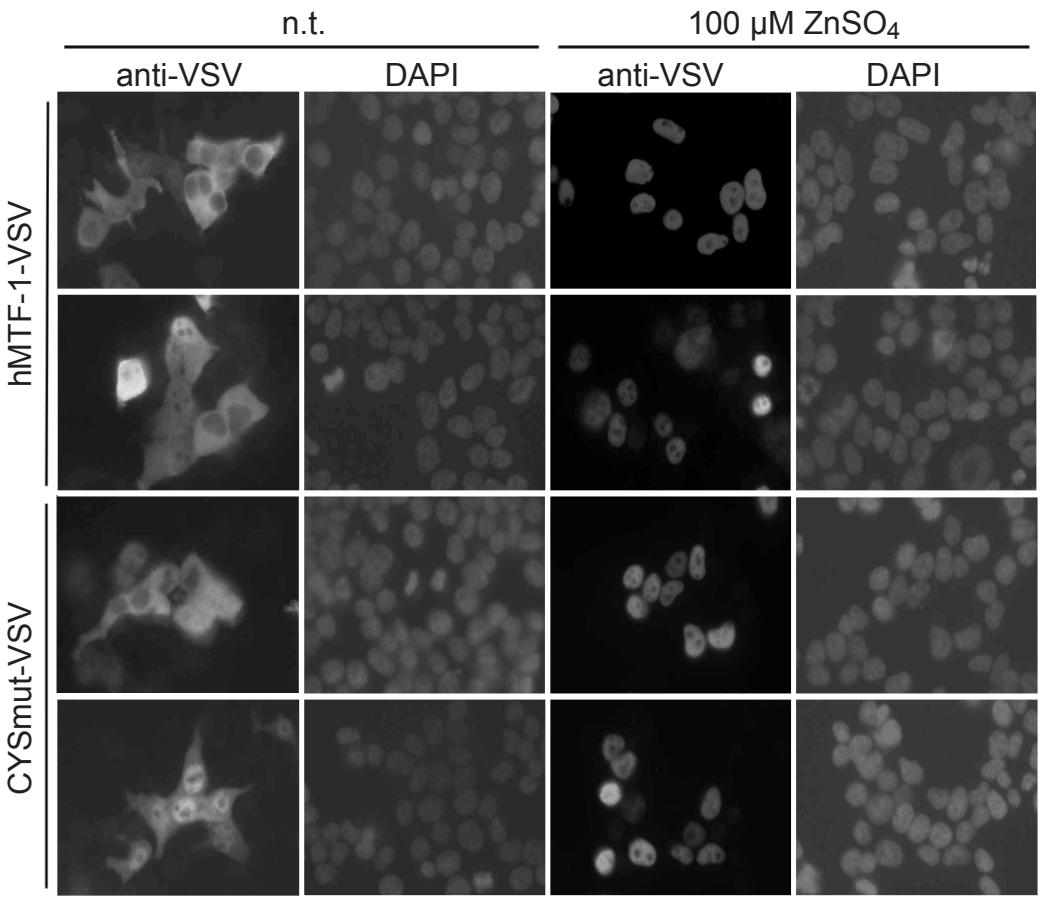

n.t.
100 µM ZnSO4
anti-VSV
DAPI
anti-VSV
DAPI
hMTF-1-VSV
CYSmut-VSV

Figure 3

C

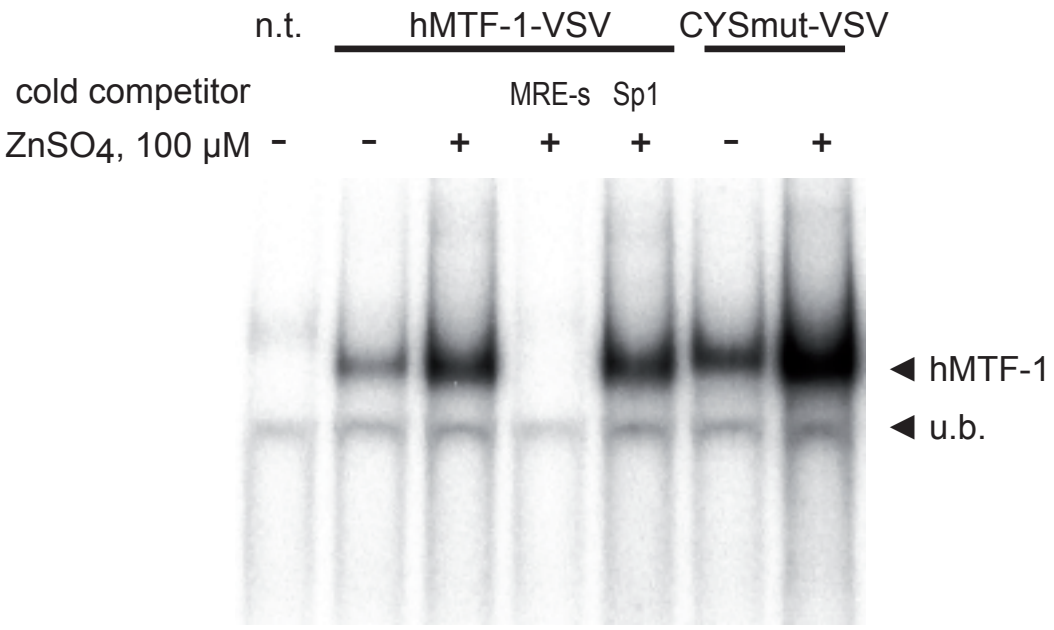

n.t.
hMTF-1-VSV
CYSmut-VSV
cold competitor
MRE-s
Sp1
ZnSO4, 100 µM
- - + + + - +
◄ hMTF-1
◄ u.b.

Figure 4

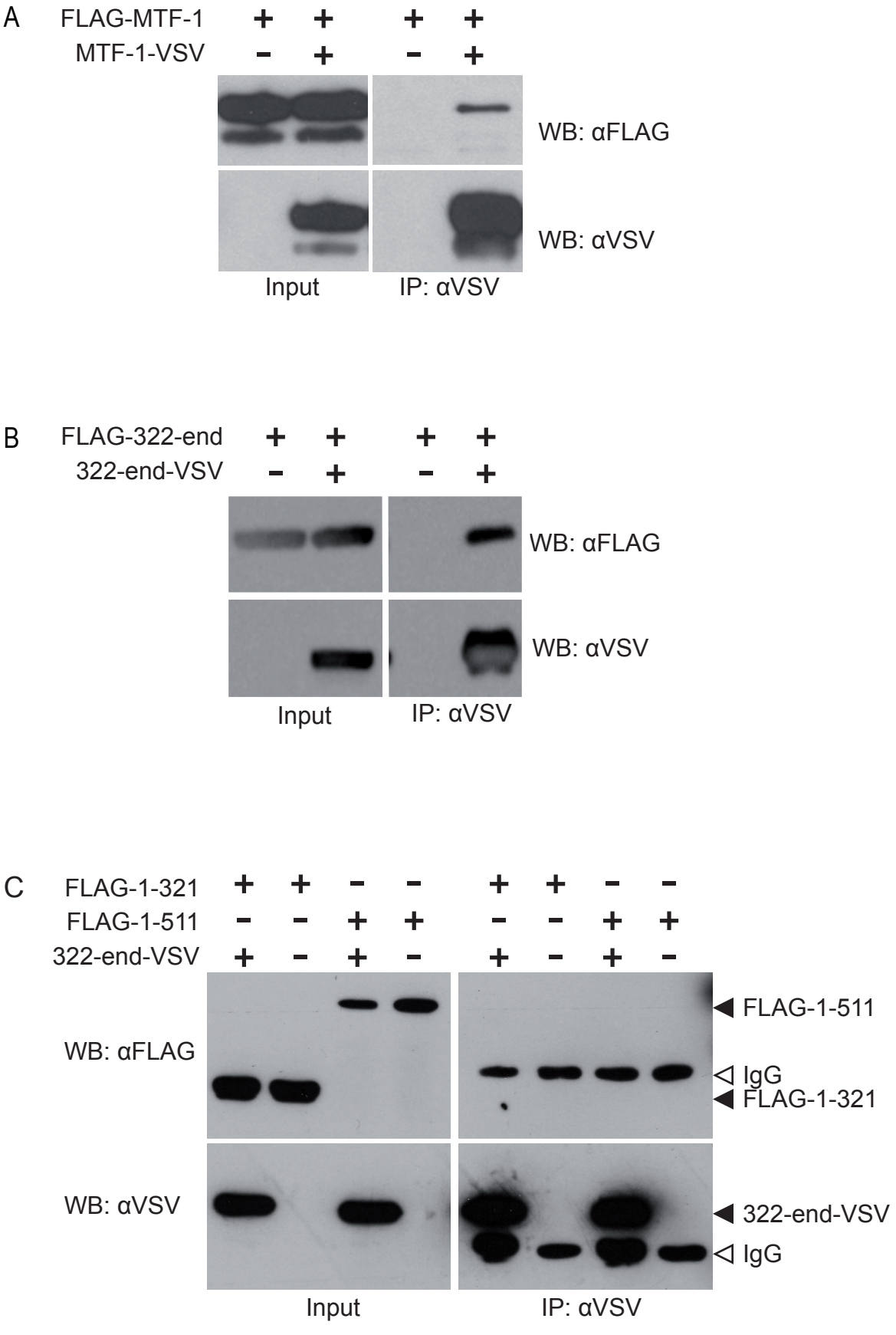
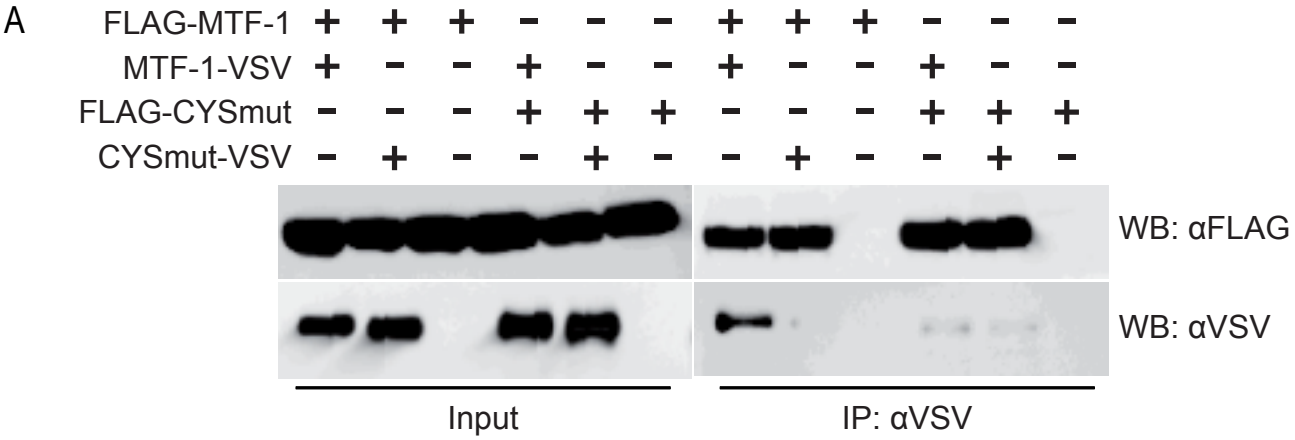
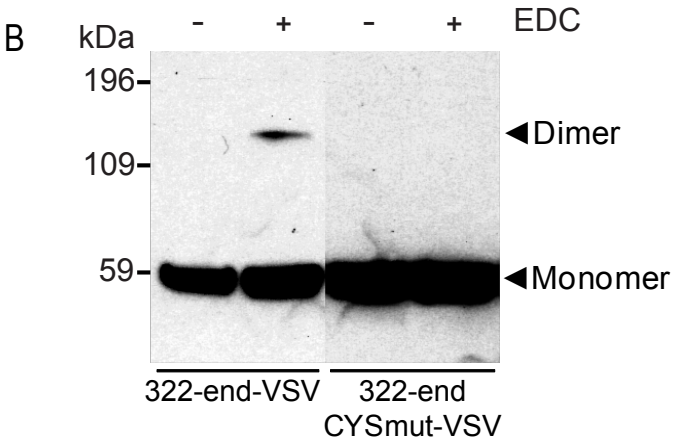

A
FLAG-MTF-1 + + + +
MTF-1-VSV - + - +
WB: αFLAG
WB: αVSV
Input
IP: αVSV
B
FLAG-322-end + + + +
322-end-VSV - + - +
WB: αFLAG
WB: αVSV
Input
IP: αVSV
C
FLAG-1-321 + + - - + + - -
FLAG-1-511 - - + + - - + +
322-end-VSV + - + - + - + -
WB: αFLAG
WB: αVSV
FLAG-1-511
IgG
FLAG-1-321
322-end-VSV
IgG
Input
IP: αVSV

Figure 5


A
FLAG-MTF-1 + + + − − − + + + − − −
MTF-1-VSV + − − + − − + − − + − −
FLAG-CYSmut − − − + + + − − − + + +
CYSmut-VSV − + − − + − − + − − + −
WB: αFLAG
WB: αVSV
Input
IP: αVSV


B
kDa
- + - + EDC
196
109
59
◀Dimer
◀Monomer
322-end-VSV
322-end CYSmut-VSV

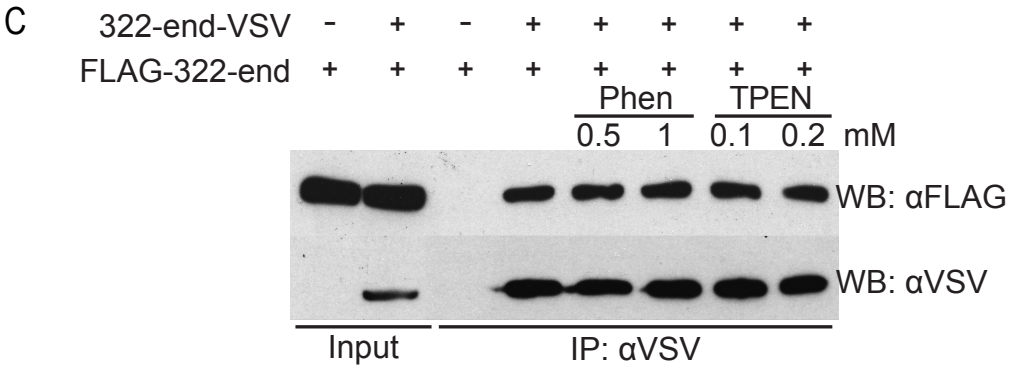

C
322-end-VSV - + - + + + + +
FLAG-322-end + + + + + + + +
Phen
TPEN
0.5 1 0.1 0.2 mM
WB: αFLAG
WB: αVSV
Input
IP: αVSV

Figure 5

D

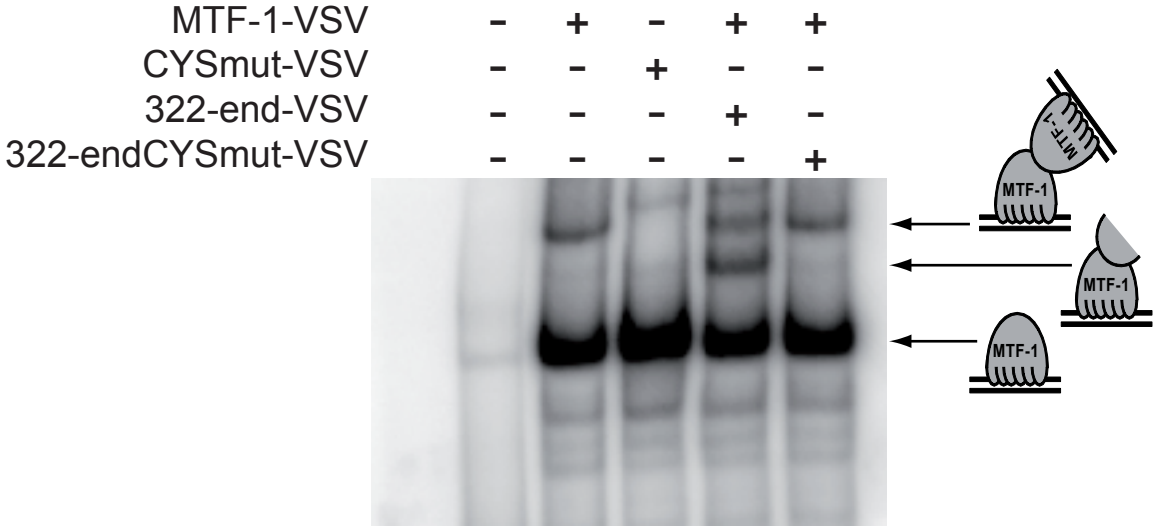

MTF-1-VSV - + - + +
CYSmut-VSV - - + - -
322-end-VSV - - - + -
322-endCYSmut-VSV - - - - +
MTF-1
MTF-1
MTF-1

Figure 6

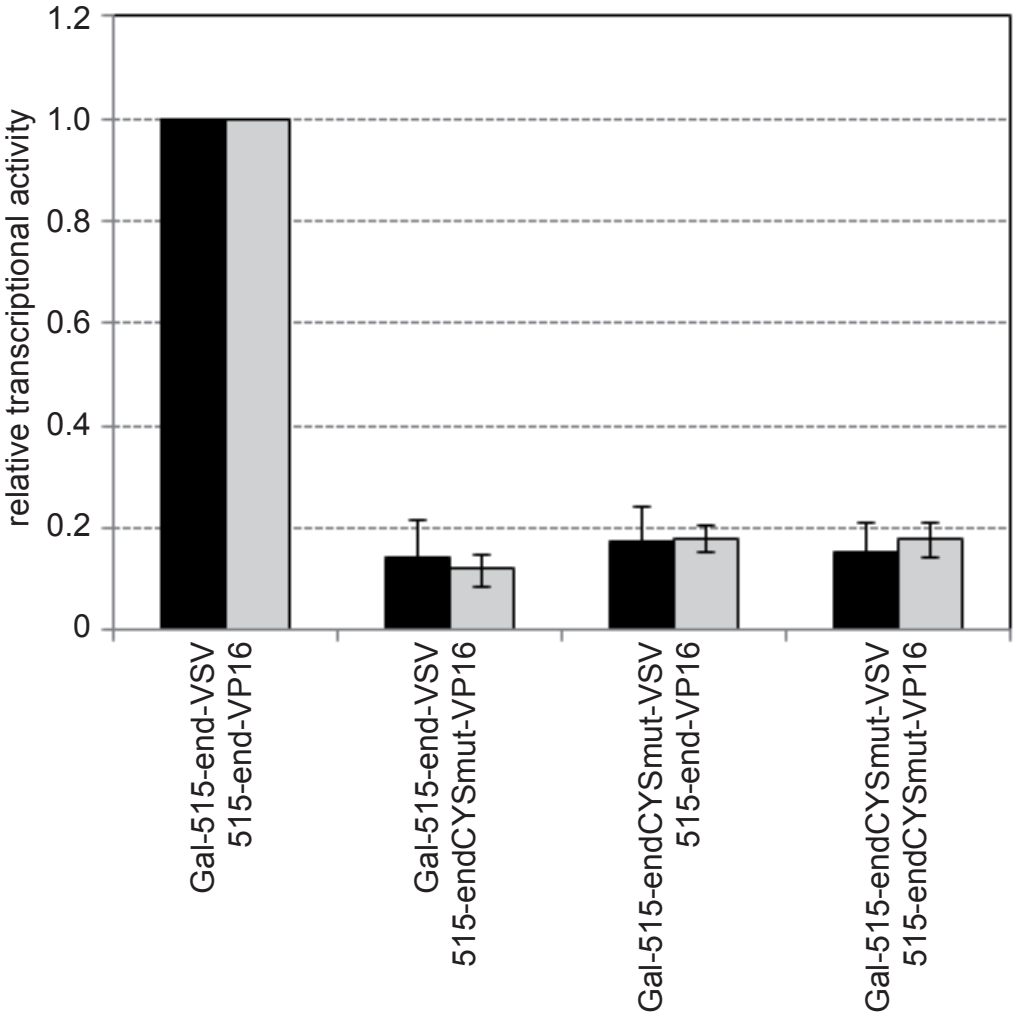
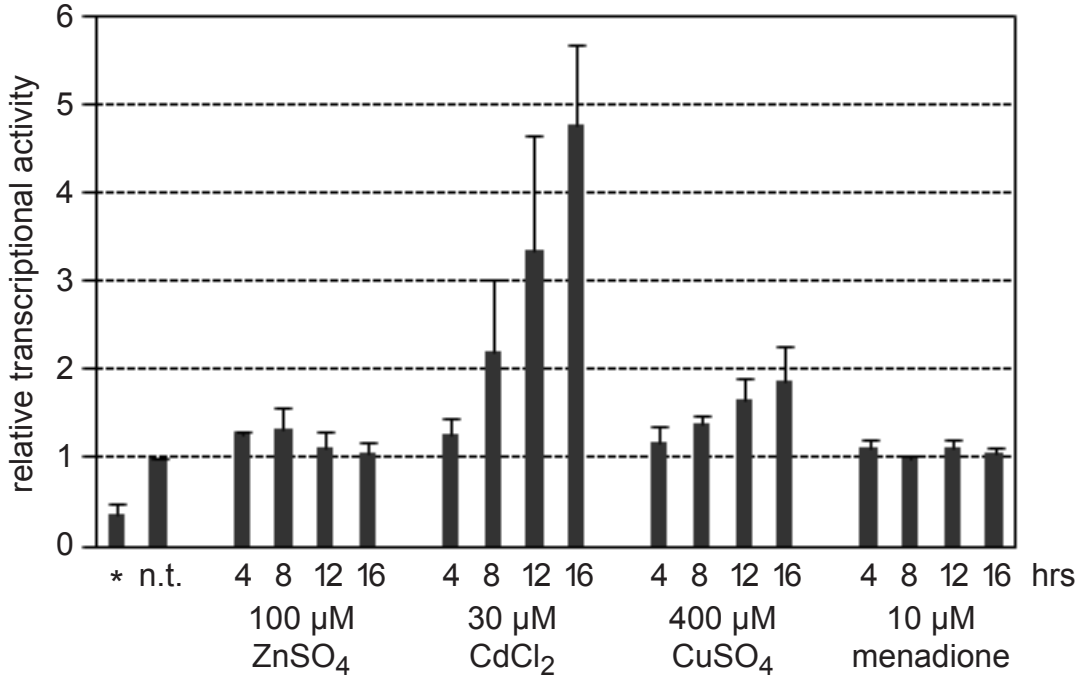
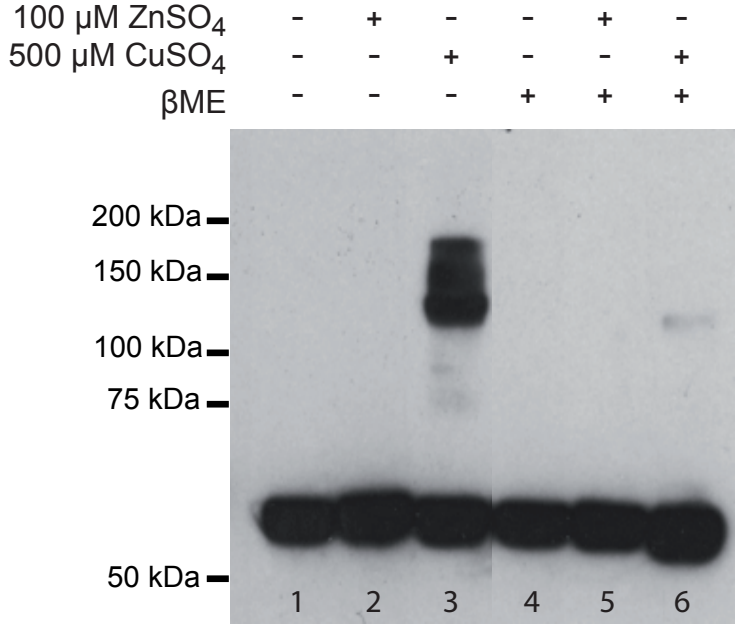

relative transcriptional activity
1.2
1.0
0.8
0.6
0.4
0.2
0
Gal-515-end-VSV
515-end-VP16
Gal-515-end-VSV
515-endCYSmut-VP16
Gal-515-endCYSmut-VSV
515-end-VP16
Gal-515-endCYSmut-VSV
515-endCYSmut-VP16

Figure 7

A


relative transcriptional activity
0
1
2
3
4
5
6
* n.t. 4 8 12 16 4 8 12 16 4 8 12 16 4 8 12 16 hrs
100 µM ZnSO4
30 µM CdCl2
400 µM CuSO4
10 µM menadione

B


100 µM ZnSO4 - + - - + -
500 µM CuSO4 - - + - - +
βME - - - + + +
200 kDa
150 kDa
100 kDa
75 kDa
50 kDa
1 2 3 4 5 6

C

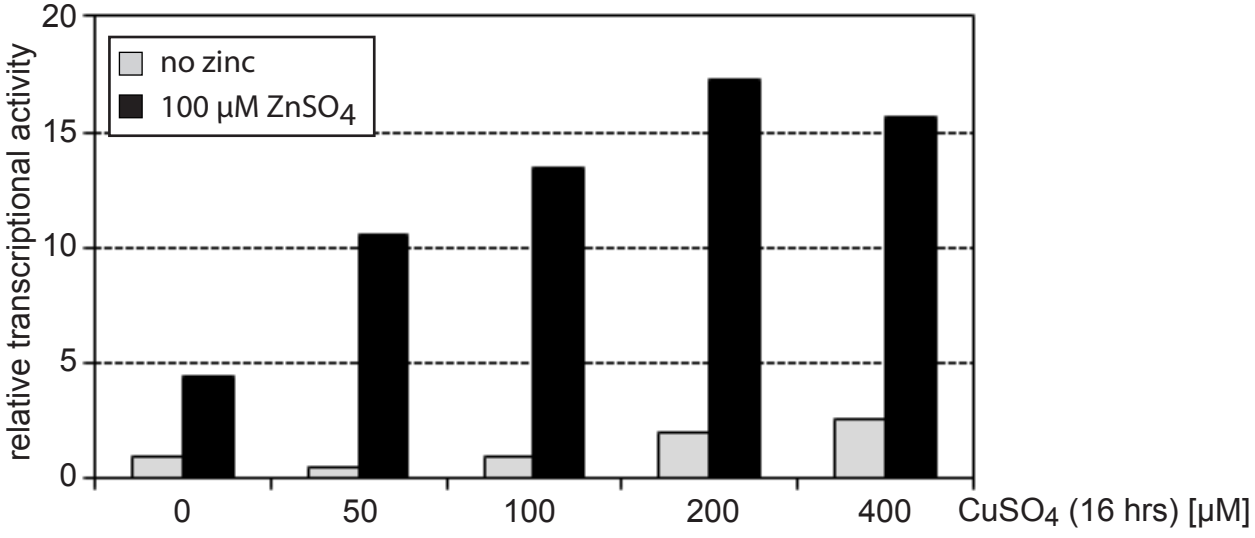

relative transcriptional activity
0
5
10
15
20
no zinc
100 µM ZnSO4
0 50 100 200 400
CuSO4 (16 hrs) [µM]

Figure 8

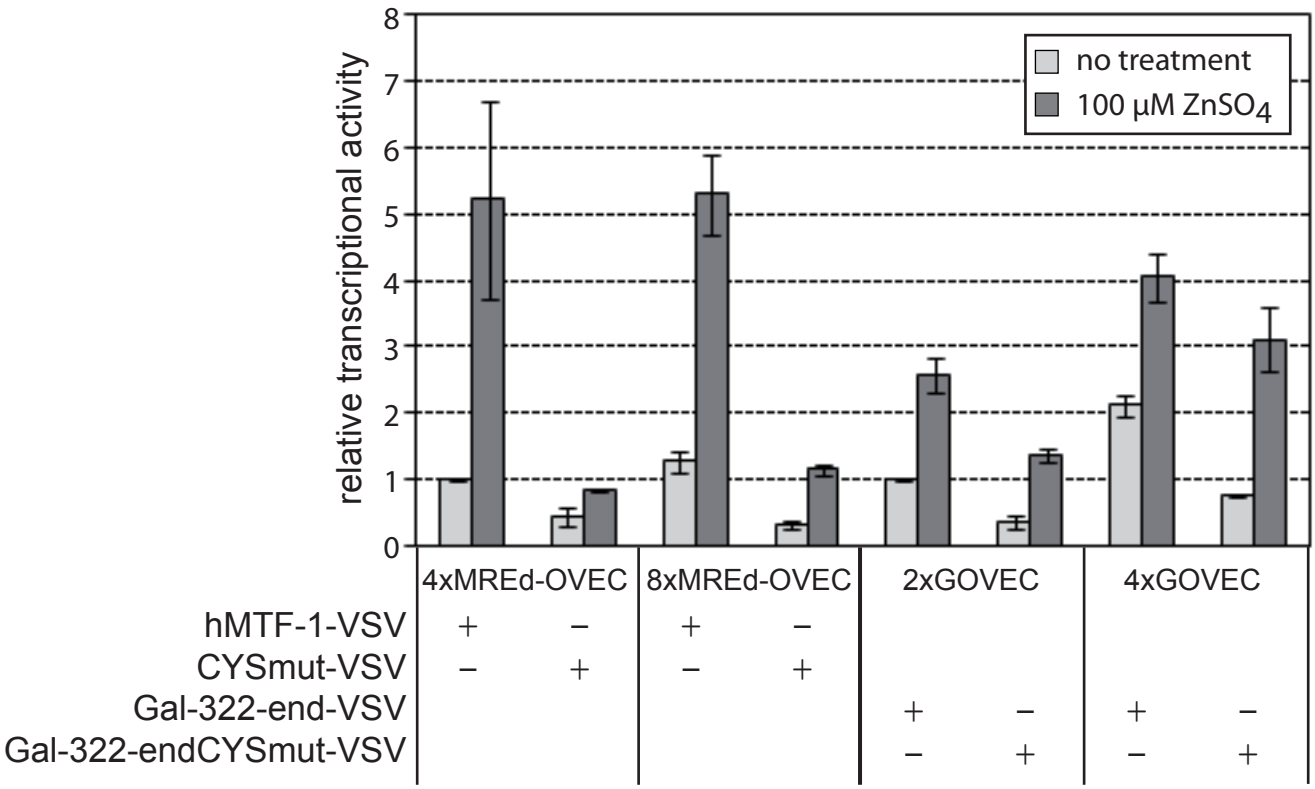
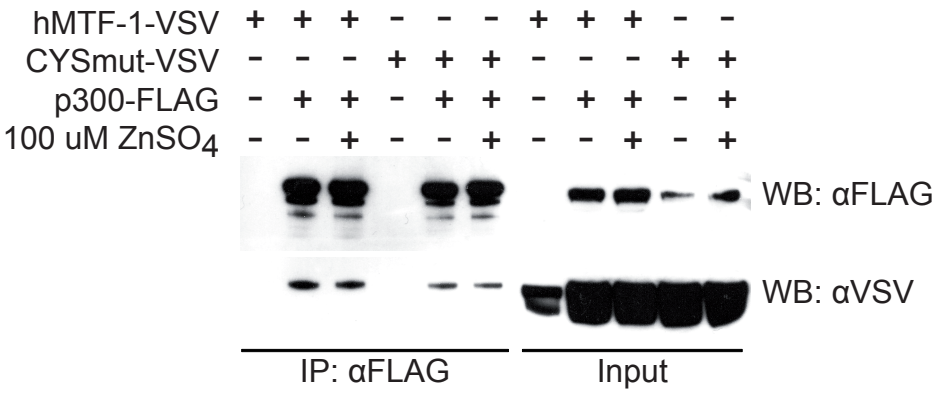

relative transcriptional activity
8
7
6
5
4
3
2
1
0
no treatment
100 µM ZnSO4
4xMREd-OVEC
8xMREd-OVEC
2xGOVEC
4xGOVEC
hMTF-1-VSV + − + −
CYSmut-VSV − + − +
Gal-322-end-VSV + − + −
Gal-322-endCYSmut-VSV − + − +

Figure 9


hMTF-1-VSV + + + - - - + + + - -
CYSmut-VSV - - - + + + - - - + +
p300-FLAG - + + - + + - + + - +
100 uM ZnSO4 - - + - - + - - + - +
WB: αFLAG
WB: αVSV
IP: αFLAG
Input

Part II: hMTF-1 interacts with, and is acetylated by, the transcriptional coactivator p300

It was shown previously that the transcriptional coactivator p300 is essential for transactivation of metallothionein gene expression mediated by mouse MTF-1 (4). Co-immunoprecipitation experiments revealed a complex consisting of mMTF-1, p300 and Sp1 that was rapidly formed upon zinc exposure. Furthermore, the interaction of mMTF-1 and p300 was shown to be dependent on leucine residues located in the acidic activation domain.

Experiments performed in our lab showed that human MTF-1, like its mouse counterpart, is interacting with p300 in a cell free system (Nurten Saydam and Walter Schaffner, unpublished). Thereby the C/H1- and the C/H3 domain of p300 were identified to mediate the interaction with hMTF-1. However, unlike the results obtained by Andrews and coworkers, GST-pulldown experiments suggested the zinc finger region rather than parts of the acidic activation domain of hMTF-1 as being important for binding p300. Subsequent *in vivo* experiments should clarify if the interface of MTF-1 with p300 differs in the mouse and human homolog. To this end, the interaction of full length hMTF-1 and p300 *in vivo* was investigated by a co-immunoprecipitation assay of VSV-tagged hMTF-1 and FLAG-tagged p300 in transiently transfected HEK293 cells. Unlike mouse MTF-1, human MTF-1 seems to constitutively interact with p300 (Figure 9, part I). This observation might be a result of overexpression, as high intracellular concentrations of the examined proteins can mask slight differences in interaction intensities.

In addition to their histone acetyltransferase activity, p300 and its close homolog CBP acetylate transcription factors and thereby modulate their function. To investigate if hMTF-1 is a target of p300 we performed an *in vitro* acetylation assay with recombinantly expressed p300 and hMTF-1 (Figure 2-1).

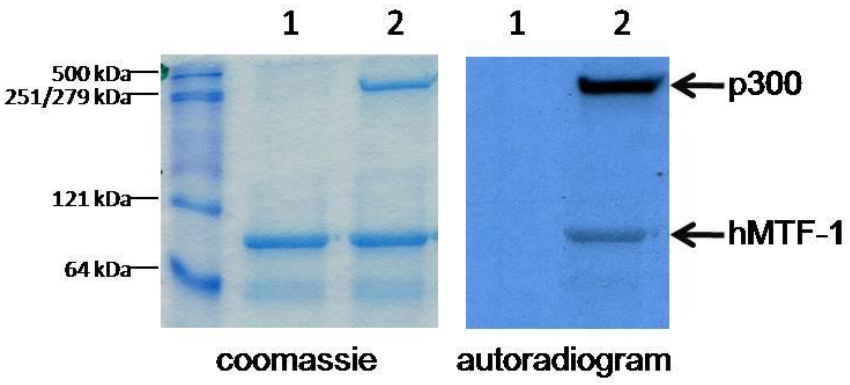


Figure 2-1: **hMTF-1 is acetylated by p300 *in vitro*.** Purified recombinant hMTF-1 was incubated with baculovirus-expressed p300 in an *in vitro* acetylation assay (lane 2). The sample shown in lane 1 was kept without p300 and serves as a negative control. Proteins were separated by SDS-PAGE and proteins were stained by coomassie (left side) prior to autoradiographie (right side). The upper band shows the autoacetylation of p300.

In this *in vitro* assay hMTF-1 is acetylated by p300 and in collaboration with the Functional Genomic Center Zürich (FGCZ) the acetylated amino acids were determined by MALDI-TOF/TOF. The sequence coverage obtained was 41.7% and four acetylated lysines could be identified, namely Lys32, Lys227, Lys268 and Lys277. However, single lysine to arginine mutations and a double mutation of Lys268 and Lys277, both located in zinc finger 5, did not alter the transcriptional activity of hMTF-1 on a 4xMREd reporter plasmid (Figure 2-2). Further analysis of a mutant in which all four lysines are mutated might reveal the importance of these acetylation sites. In addition, changes in transcriptional activity might only become

apparent if the expression of endogenous and fully chromatinized MTF-1 target genes is analyzed.

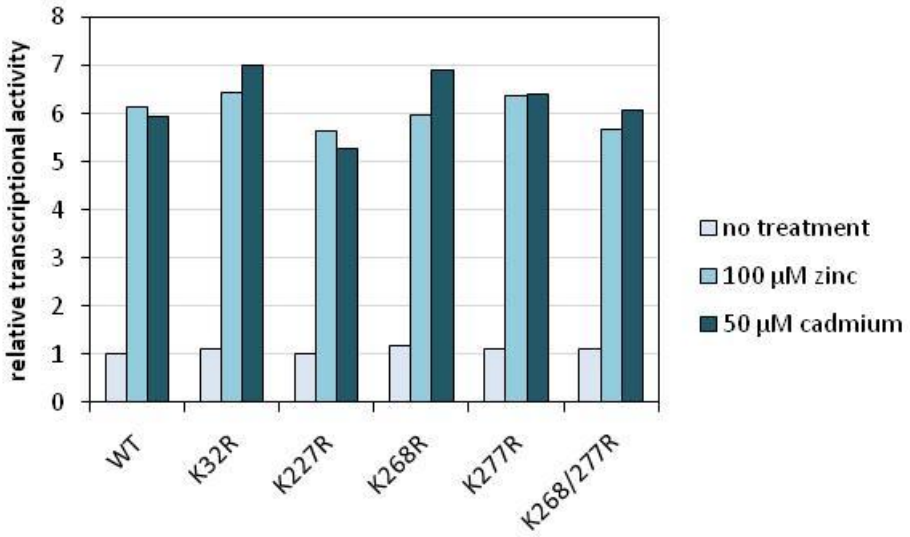


Figure 2-2: **The transcriptional activity of hMTF-1 is not impaired by mutation of potential acetylation sites.** MTF-1-/- cells were transfected with wild type or mutated MTF-1 expression clones, the 4xMREd reporter and a reference plasmid. Transcripts were quantified by the S1-nuclease protection assay.

Acetylation by p300/CBP can either enhance or repress the activity of a transcription factor. We used sodium butyrate (NaBu) to inhibit histone deacetylase (HDAC) activity and thereby stabilize protein acetylation. Low concentrations (0.1 – 1.0 mM) of NaBu did not alter the expression of a 4xMREd-drived reporter gene, but treatment with 2.5 or 5 mM NaBu clearly increased zinc-induced reporter gene expression, whereas basal activity remained unchanged (Figure 2-3). This result supports a role for acetylation in metal-induced transcriptional activity of hMTF-1. Further studies with anti-acetylated-lysine antibodies might reveal whether or not hMTF-1 is really hyperacetylated upon metal induction.

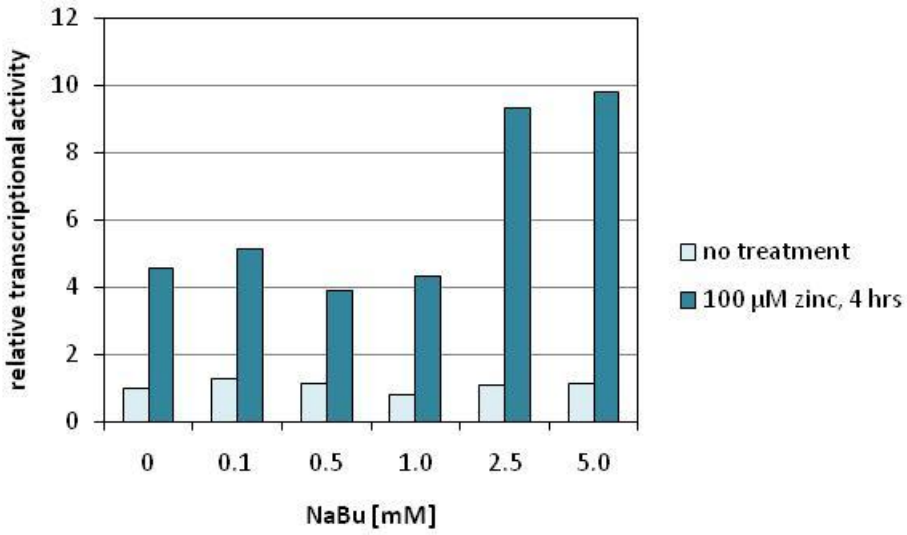


Figure 2-3: **Effect of the HDAC inhibitor sodium butyrate (NaBu) on the transcriptional activity of hMTF-1.** MTF-1-/- cells were transfected with an hMTF-1 expression plasmid, the 4xMREd reporter and OVEC-Ref reference plasmid and treated with NaBu for 48 hrs with the indicated concentrations prior to zinc induction and cell harvest. Reporter gene expression levels were quantified using the S1-nuclease protection assay.

Materials and Methods to part II

Cell culture and transfections

HEK293 and MTF-1 -/- (dko7 (6)) cells were maintained in Dulbecco´s Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and 8 % fetal bovine serum (FBS; Biochrom AG, Berlin). Transient transfections were performed in 10 cm cell culture dishes with the standard calcium phosphate co-precipitation method. To equalize transfected DNA amounts herring sperm DNA was added to a total amount of 20 µg of DNA.

Plasmid constructions

The plasmid expressing p300-FLAG under the control of the CMV-promoter was a generous gift of Prof. Michael Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, UZH). The plasmid expressing a VSV-tagged hMTF-1 cDNA clone (pC-hMTF-1VSV) was described previously (8) and single amino acid substitutions were generated by site-directed mutagenesis (QuikChange, Stratagene) according to manufacturer's instructions. The detailed cloning procedures and primer sequences are available on request. The reporter plasmid 4xMREd-OVEC and the reference plasmid OVEC-Ref were described previously (7, 12).

S1-nuclease protection assay

Isolation of RNA and the S1-nuclease protection assay were performed as described previously (11, 12). Signals were measured using the image analyzer FLA-700 and quantified using ImageGauge software (Fujifilm life science). Reporter signals were normalized to the reference signal and values of untreated wild type samples were set to 1.

In vitro acetylation assay

The *in vitro* acetylation assay with radiolabeled Acetyl-CoA was basically performed as described in (3) using 0.5 µg of baculovirus-purified p300 and 2 µg of recombinant hMTF-1. The baculo-purified p300 was a generous gift of Prof. Michael Hottiger (Institute of Veterinary Biochemistry and Molecular Biology, UZH). Proteins were separated by SDS-PAGE and stained by Coomassie. Dried gels were subjected to autoradiography to visualize acetylated proteins.

Expression of recombinant hMTF-1

Rosetta^{TM} cells expressing an intein/chitin binding domain-tagged hMTF-1 cDNA clone (pTYB1 expression vector, NEB) were dissolved and lysed in a buffer containing 200 mM Tris pH 8.0, 10 % Glycerol, 0.1 % Triton X100, 1 mM EDTA pH 8.0, 500 mM NaCl and protease inhibitors ("chitin buffer"). Recombinant hMTF-1 was immobilized on chitin beads and washed with chitin buffer. For elution, beads were incubated with chitin buffer supplemented with 0.1 M DTT over night, which induces self-cleavage of the recombinant fusion protein from the intein moiety.

Part III: Functional domains of the *Drosophila* homolog of metal-responsive transcription factor-1

In order to further characterize the functional domains of the *Drosophila* homolog of MTF- 1 we generated several mutants (Table 3-1) that were introduced into the fly genome via a phiC31-based system, which allows site directed insertion of a transgene into an AttP site at defined genomic loci (1). In further steps these transgenes will be crossed into the dMTF-1 knockout background which will reveal their functionality. In parallel, these expression clones will be tested in *Drosophila* cell culture with an OVEC-based reporter gene assay (12).

dMTF-1 mutation	**Comments**
Tyr118Ala	Potential phosphorylation site
Ser126Ala	Potential phosphorylation site
Thr127Ala	Potential phosphorylation site
Ser160Ala	Potential phosphorylation site
760stop	Truncation mutant missing amino acids 761-791
636stop	Truncation mutant missing amino acids 637-791
579stop	Truncation mutant missing amino acids 580-791
CYSall	Cys→Ala mutations of all cysteines of the "metallothionein" like region

Table 3-1: dMTF-1 mutations that will be tested in *Drosophila* and cell culture.

Preliminary results indicate that the expression of 636stop and 579stop truncation mutants have a strong phenotype on an otherwise MTF-1 wild type background, namely decreased eclosure and survival rate, female sterility and a severe wing phenotype (Figure 3-1). The 760stop mutant shows a mild wing phenotype, is fertile and has a slightly decreased life span.

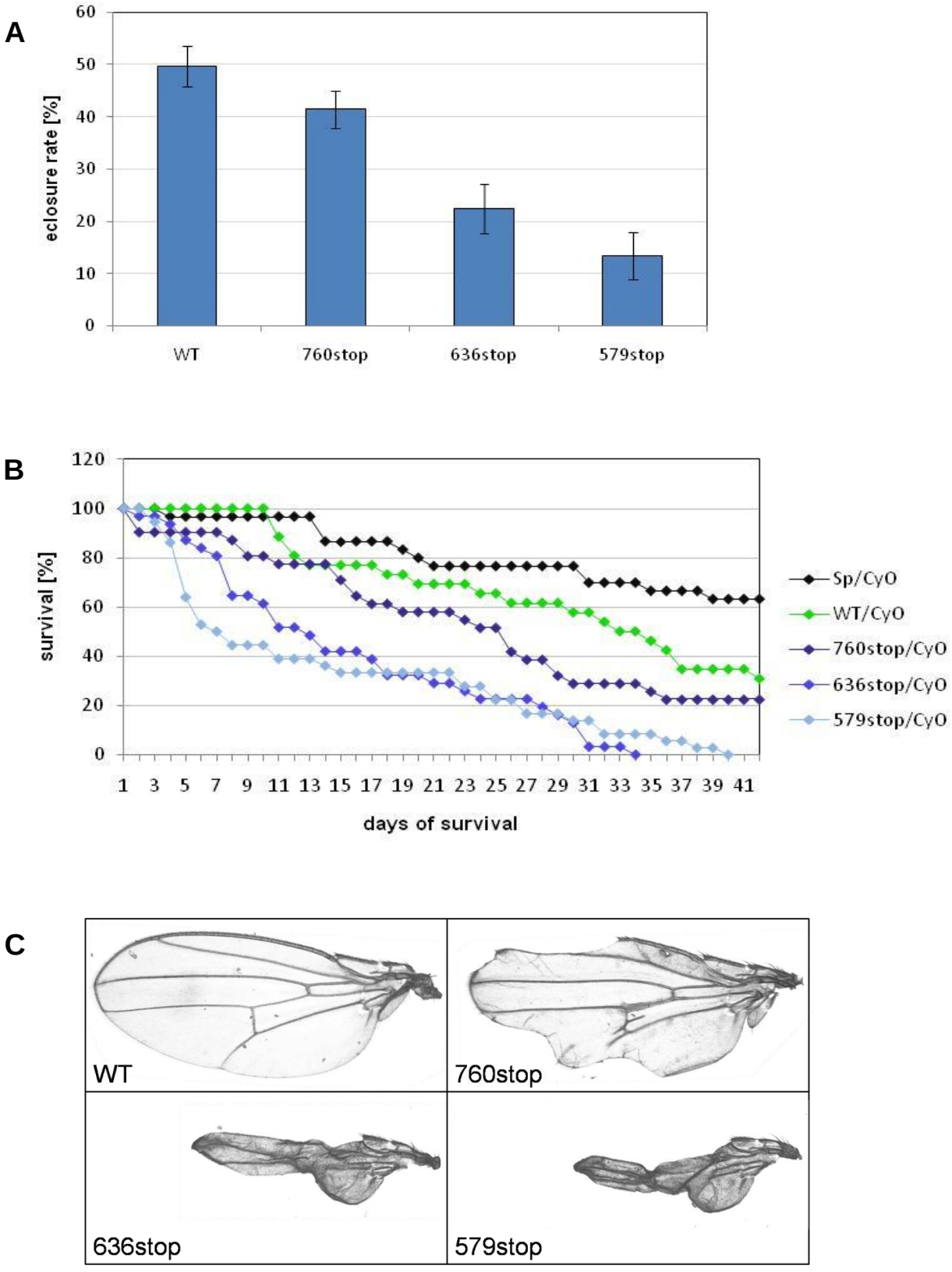


Figure 3-1: **The truncation mutants show a reduced eclosure and survival rate and a disturbed wing development.** (A) Heterozygous flies expressing the indicated dMTF-1 clones were crossed to y w flies and the rate of construct carrying flies (marked by the white gene) were counted. Error bars indicate standard deviations of three independent experiments. (B) Approx. 30 heterozygous virgins were kept at 25 °C and surviving animals were counted each day. (C) Wings of transgenic flies expressing the indicated constructs in a y w background.

In a first approach three potential NES motifs were found according to their high content of hydrophobic amino acids (Figure 3-2). These potential motifs were fused in-frame into a vector containing two tandem copies of GFP and the subcellular localization of the fusion proteins was analyzed in transiently transfected HEK293T cells.

NES0: 406-KPIIPMAPLTDACVALPTEMPSFVNLK-432

NES1: 611-TEMNQNIEDVALLLQNLASMS-631

NES2: 761-CCVVICIKTLQALRKVLRT-779

Figure 3-2: Potential NES domains that were tested in HEK293T cells. Large, hydrophobic residues are highlighted in red.

Due to its size 2xGFP is equally distributed to the nucleus and the cytoplasm as it can freely pass the nuclear pore. Fusion of NES0 did not change the localization of 2xGFP, but both, NES1 and NES2, conferred nuclear exclusion to the fusion protein (Figure 3-3).

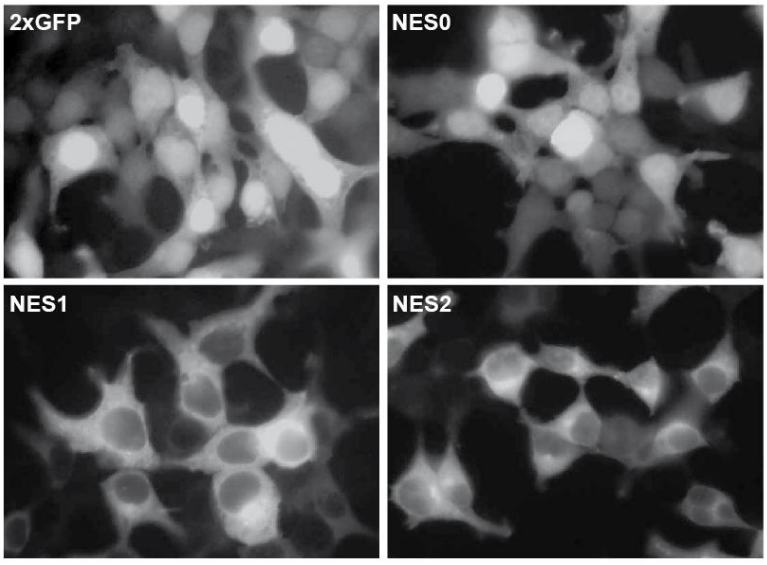


Figure 3-3: **NES1 and NES2 confer nuclear exclusion to a 2xGFP fusion protein**. HEK293T cells were transfected with 2xGFP-fusion clones containing potential NES sequences. The exposure time was kept at 150 ms for 2xGFP, NES0 and NES1 fusions and at 500 ms for NES2 due to low signal intensity.

In addition, the NES2-fusion protein signal is weaker as compared to the other proteins tested. This might reflect a lower protein stability due to aggregation and subsequent degradation via the proteasome, or these amino acids encode a specific protein degradation signal which is important to restrict dMTF-1 levels in the cell. Interestingly, this domain is also found in other insects, notably in the C-terminus of honeybee MTF-1, which is otherwise not very similar to dMTF-1, suggesting an important role of this region.

A sequence motif rich in basic amino acids (^{292}KGRKKRPLK^{300}) which resembles the classical nuclear-localization signal (NLS) might confer nuclear localization to dMTF-1. In the case of human MTF-1 the sequence that matches the classical NLS only plays a minor role in mediating nuclear accumulation. Rather, zinc fingers 1-3 were most important in tests with deletion mutants and fusion proteins (5). To see if the same is true for dMTF-1 we will test fusions of dMTF-1 fragments to the myc-tagged chicken pyruvate kinase, which was tested to be solely cytoplasmic and thus a suitable tool to study potential NLS-domains (9). To locate the transcriptional activation domain of dMTF-1, subsegments of the C-terminal half were fused to the Gal4 DNA-binding domain and the activity of these constructs will be determined in *Drosophila* cell culture.

Materials and Methods for part III

Fly stock keeping and generation of transgenic flies

One liter of fly food contains 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent Nipagin (15% in ethanol). Flies were raised and kept at 25 °C and 60% humidity. Plasmids were introduced into the fly genome at an AttP landing site located at the genomic position 51D using the phiC31 integration system (1).

Cell culture and transfections

HEK293T cells were kept in Dulbecco´s Modified Eagle Medium (DMEM, Gibco) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Invitrogen) and 8 % fetal bovine serum (FBS; Biochrom AG, Berlin). For testing the NES-2xGFP fusion proteins, cells were split on cover slips in a 12-well cell culture plate and transfected with the standard calcium phosphate co-precipitation method.

Plasmid constructions

An α-tubulin-promoter driven, VSV-tagged cDNA clone of dMTF-1 was cloned into the pAttB-vector and modifications to create the respective mutants were generated based on this vector by site-directed mutagenesis (QuikChange, Stratagene) or common PCR- and restriction enzyme-based cloning strategies. Sequences of the required oligonucleotides and detailed cloning procedures are available on request.

Distorted copper homeostasis and (high) cisplatin resistance upon chaperone Atox1 deletion in *Drosophila*

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Abstract

Copper is an integral part of several proteins and thus an essential trace metal. However, due to its redox activity which leads to radical generation, it can also be highly toxic and every cell has to carefully control its bioavailability. Eukaryotes contain three copper chaperones, Atx1/Atox1 which delivers copper to ATP7 transporters located in the trans-Golgi network, Cox17 which provides copper to the mitochondrial cytochrome c oxidase, and CCS which is a copper chaperone for superoxide dismutase 1. Here we describe the knockout phenotype of the *Drosophila* homolog of mammalian Atox1 (Atx1 in yeast). Atox1-/- flies develop normally, though at reduced numbers, and the eclosing flies are fertile. However, the mutants do fail to develop on low-copper food.

We find that the intestinal copper importer Ctr1B, which is regulated by copper demand, fails to be induced upon copper starvation in Atox-/- larvae. Copper accumulates in the intestinal cells of mutant larvae, but cannot be delivered to the DmATP7 protein which transports copper into the trans-Golgi network and is also responsible for copper export from the gut to the rest of the body. In addition, compared to controls, *Drosophila* Atox1 mutants are more resistant to the anticancer drug cisplatin, a compound which is also imported via Ctr1B.

Introduction

All organisms, including eukaryotes from yeast to humans, use elaborate systems to regulate copper homeostasis [1-5]. Copper is a trace metal that is both indispensable for normal cell function and potentially toxic. Due to its capacity to undergo redox cycling between Cu(I) and Cu(II), copper serves as a cofactor of redox enzymes such as Cu/Zn-superoxide dismutase (Cu/Zn SOD), tyrosinase, lysyl oxidase and cytochrome c oxidase (COX), whose functions are compromised by copper deficiency. Several diseases are characterized by an aberrant copper metabolism. In Menkes disease, mutations in the copper transporter ATP7A gene cause an insufficient peripheral supply of copper [6]. A defective copper homeostasis has also been implicated in Alzheimer's disease (AD), where patients were reported to have a reduced copper content in the brain [7-9].

In all eukaryotes, copper is imported as Cu(I) by high-affinity copper transporters of the Ctr family [10]. Ctr importers form homotrimeric complexes in the membrane and acquire copper in an ATP independent manner [11, 12]. In the rapidly growing *Drosophila* larvae, copper import from the food is mainly executed by the copper importer Ctr1B [13-15]. *Drosophila* has two additional copper importers; Ctr1A is ubiquitously expressed at all stages of development at a relatively low level [16] and Ctr1C, which is strongly expressed in the male gonads. Ctr1C, in cooperation with Ctr1B, ensures adequate copper supply for germ cell formation and fertility (D. Steiger and W.S., unpublished).

There are three types of copper chaperones that accept copper from Ctr proteins and transfer it to their specific target proteins [17]: CCS, which delivers copper to SOD1 (Cu/Zn superoxide dismutase) [18-20], Cox17, which transfers copper to

mitochondrial cytochrome c oxidase [21, 22], and Atox1. In yeast, Atx1 brings copper to Ccc2, a copper transporting ATPase [23]. Atox1, the human ortholog of yeast Atx1, delivers copper to the human Ccc2 homologs, ATP7A and ATP7B, which localize to the trans-Golgi network [24, 25]. The *Drosophila* ortholog of yeast Ccc2 and mammalian ATP7A and ATP7B genes, designated DmATP7, has been characterized by J. Camakaris, R. Burke and colleagues [26, 27]. It delivers copper to cuproenzymes and also serves to export excess cellular copper; accordingly, DmATP7 mutants overaccumulate copper in the intestine but suffer from copper deficiency in other parts of the body.

Atox1 is very well conserved among eukaryotes, from mammals to insects and fungi (Fig 1A). A homolog of Atox1, called CopZ, was also identified in bacteria [28]. In mice, lack of Atox1 leads to copper deficiency with a high mortality shortly after birth. Surviving animals display a severe phenotype that includes growth failure, skin laxity and hypopigmentation [29]. In the absence of Atox1, copper accumulates in cultured mouse fibroblasts due to impaired copper efflux [25]. Since the molecular function of *Drosophila* Atox1 has not been elucidated so far, we decided to generate an Atox1 null mutant. Our results show that Atox1 mutant flies are sensitive to copper starvation but, interestingly, more resistant to cisplatin treatment, a drug that is imported via Ctr1 type transporters [30, 31]. In the Atox1 mutant, probably analogous to the DmATP7 mutant, copper accumulates in intestinal cells due to inefficient efflux of copper to the rest of the body.

Materials and methods

Fly stock maintenance

One liter of fly food is composed of 55 g corn, 10 g wheat, 100 g yeast, 75 g glucose, 8 g agar and 15 ml anti-fungal agent nipagin (15% in ethanol). Flies were raised and experiments were performed at 25ºC and 60% humidity.

Generation of the Atox1 null allele

An Atox1 null allele ($Atox1^{174}$) was generated by imprecise excision of a single P-element insert 30 bp upstream of the transcription start site in the EP line EY15780 (BL21158, Bloomingtion Stock Center), which turned out to be a hypomorph. EP flies were crossed to a line containing a transposase (y w; ; Δ2-3 Sb/TM2, a gift from Konrad Basler). 200 excision events were analyzed by PCR followed by sequencing. Among them, a 450-base pair deletion ($Atox1^{174}$) beginning at the EP insertion site was identified, which removed the first two of the three exons of the Atox1 gene, whereas neighboring transcription units were not affected. A precise excision without any deletion was also generated, and served as the wild type Atox1 control for genetic background in all experiments.

Survival assay

For survival experiments, fly medium was supplemented with CuSO4,

bathocuproinedisulfonic (BCS) acid disodium salt (Sigma B1125) or cis-diammineplatinum(II) dichloride (Sigma P4394) to the indicated concentrations. BCS is a metal chelator with a strong preference for Cu(I). To measure the survival rate, 100 eggs were transferred to a vial containing standard food (NF) or to food with the indicated supplements. The survival rate was calculated as the percentage of flies that eclosed from the eggs. Each bar represents the average of at least three vials.

Genomic rescue construct and an Atox1 overexpression fly line

To generate a genomic rescue construct, the genomic region of the Atox1 gene including 300 bp upstream of the transcription start and 2 kb downstream of the transcription unit was cloned into a pAttB vector. The Atox1 coding sequence tagged with mCherry at the C-terminus was cloned into the transformation vector pUAST-AttB. Plasmids were introduced into the *Drosophila* genome at an AttP landing site (line ZH-51D) using the Phi C31 integration system [32]. The resulting transgenic flies are designated Atox1-Res, Atox1-Res-HA and UAS-Atox1-mCherry. For expression of the Atox1-mCherry protein, the UAS-Atox1-mCherry flies were crossed with the flies containing the transactivator Gal4 driven by the ubiquitously active *actin 5c* promoter (*actin-Gal4*).

Fluorescent microscopy

Guts were dissected from Atox1-mCherry adult flies and fixed by 4% formaldehyde for 20 min. Confocal images were recorded on a Zeiss LSM710 microscope. The images were recorded at 10x magnification and with 5 seconds exposure time.

To visualize the expression level of the MtnB-YFP reporter gene [33] guts of 1-2 day old female flies were dissected and YFP expression was recorded with a Zeiss Axioplan microscope and a Zeiss Axioplan MRm camera without fixation of the tissue. To distinguish yeast autofluorescence in the gut from specific YFP signal, images of the FITC and TRITC filter were merged digitally using ImageJ software. Exposure times were kept the same for all recordings.

S1 nuclease protection assay

To determine mRNA levels, 3rd instar larvae were collected on either standard food or food with the indicated supplements. Total RNA was extracted using the TRIzol reagent (Life Technologies) and nuclease S1 mapping of transcripts was performed as described previously [34]. The gels were developed using FLA-7000 system and bands were quantified using ImageGauge software (Fuji Film). The transcripts of the endogenous actin 5c gene were measured and used for normalization of transcript levels.

Results

Atox1 mutant flies are sensitive to copper starvation

The *Drosophila* homolog of human Atox1 was identified previously [35]. To analyze its molecular function in more detail we generated a null mutant allele (Fig 1B). Starting from a *Drosophila* strain with an insert of a modified P-element in the Atox1 promoter region (EP line EY15780), we attempted to obtain deletion mutants of the Atox1 gene by imprecise "jump-out". Indeed, among 200 excision events, one was found with a deletion of most of the transcription unit ($Atox1^{174}$) and others with larger or smaller deletions. There were also several precise excisions, one of which was used as a control for the genetic background. The level of Atox1 transcripts in control larvae maintained on normal food (NF), on copper-containing food and on copper-depleted food brought about by the copper chelator BCS was determined. The Atox1 mRNA level was also measured in the EP line and in the $Atox1^{174}$ deletion mutant flies (Fig 2A). The transcript levels of wild type Atox1 were unaffected by copper-supplemented food (250 µM $CuSO_4$) or by copper-deprived food (100 µM BCS). In the EY15780 line, which has a P-element insertion in the Atox1 promoter region, there was a dramatic reduction of Atox1 transcripts to only 10% of controls. In $Atox1^{174}$ deletion mutant flies no Atox1 transcripts above background level could be detected. Atox1 mutant flies are viable and fertile under standard laboratory conditions, although the survival rate during development is lower than that of wild type flies. The most obvious phenotype is that Atox1 flies cannot tolerate copper starvation under conditions, which pose no problem for wild type *Drosophila* (Fig 2B).

Expression patterns of tagged Atox1

To detect the endogenous expression pattern of Atox1 in *Drosophila*, a transgene with the genomic Atox1 gene was generated, in which a hemagglutinin (HA) tag was fused to the C-terminus of Atox1. This construct was integrated into the Drosophila genome via the phage C31 integration system at an AttP site located at genome position 51D and the expression pattern was visualized by immunostaining. In the adult gut, Atox1 is strongly expressed in enterocytes and another cell type which most likely represents intestinal stem cells, as inferred from their distribution and low degree of ploidy, typical for cells that are able to divide (data not shown). In another construct, a fusion protein with the fluorescent protein mCherry was expressed under the control of the ubiquitous transactivator actin-Gal4. In enterocytes of the gut, the Atox1-mCherry protein yielded strong fluorescence with a punctate expression pattern (Fig 3). By contrast, Atox1-mCherry levels were much lower when the flies were exposed to copper-containing food. In contrast to the situation in enterocytes, Atox1 levels were not affected by copper status in intestinal stem cells. This suggests that regulated expression is particularly important in enterocytes, since these are the cells that have to cope with fluctuating food copper levels.

Copper accumulates in the gut of Atox1 mutant larvae and adult flies

The major copper importer in the larval gut, Ctr1B, is regulated at the transcriptional level: transcripts are induced at low copper conditions and repressed below basal level

under conditions of excess copper [14, 15]. We used the S1-nuclease protection assay to monitor the transcriptional response of Ctr1B (Fig 4A). While Ctr1B is up-regulated by copper starvation in wild type flies, in Atox1 mutant flies Ctr1B transcripts remained low upon growth in BCS-supplemented food. Additionally the expression of a MtnB-YFP reporter gene is stronger in Atox1 mutant flies (Fig 4B), supporting the idea that, in the absence of the specific chaperone, copper export via DmATP7 from intestinal cells to the rest of the body is impaired, with concomitant copper accumulation in the gut (see Discussion).

Atox1 mutant flies are resistant to cisplatin treatment

Cisplatin is a widely used anticancer drug, which after its uptake into cells is able to covalently bind to DNA, causing DNA replication block and apoptosis of target cells [36]. It was previously found that copper importers of the Ctr type are also major importers of cisplatin, even though the compound is structurally unrelated to copper ions. However, cisplatin uptake is apparently not executed via the same mechanism as copper import [37]. Since in mice fibroblasts loss of Atox1 leads to an increased resistance to cisplatin [38], we also determined the sensitivity of Atox1 mutant flies to cisplatin. Whereas wild type flies show a significantly reduced survival to adulthood on food containing 1 mM cisplatin, the viability of Atox1 mutants was not affected by cisplatin at the same concentration (Fig 5).

Discussion

Starting out with a P-element insertion in the upstream promoter region of the copper chaperone Atox1, we generated a null allele by imprecise excision. The fact that such flies are viable, although with a lower eclosion rate than control flies, and fertile shows that the Atox1 gene is not strictly required under standard laboratory conditions. However, our results clearly reveal a distorted copper homeostasis in *Drosophila* lacking Atox1. While the mutants are not affected by copper load, they are highly sensitive to copper depletion: no survivors were ever recorded at 250 µM BCS, a concentration which is readily tolerated by control flies, either Atox1 wild type flies or Atox1 mutants rescued with an Atox1 transgene. Atox1 is particularly abundant in the so called "copper cells" in the midgut of both larvae and adult flies. These cells can accumulate excess amounts of copper upon copper load and readily lose their store upon copper depletion, apparently by transport via DmATP7 into the rest of the body [13]. While Atox1 transcripts are hardly, if at all, changed by copper status of the flies, the Atox1 protein level was reduced upon copper load: in transgenic flies expressing Atox1-mCherry protein, Atox1 forms a strong punctate staining in enterocytes, while staining is dull upon copper load. This indicates a posttranscriptional regulation, possibly at the level of translation efficiency and/or protein stability. To probe the possible interplay of Atox1 with the copper importer Ctr1B, or with the metalloregulatory transcription factor MTF-1, we also analyzed Ctr1B and Atox1 double mutants, and MTF-1 and Atox1 double mutants. However, at least in normal food we did not observe any change in phenotype with either of these double mutants flies (data not shown).

The fact that expression of the major intestinal copper importer, Ctr1B, is not induced by copper depletion in the Atox1 null mutant suggests that the intestinal cells have accumulated enough copper to exert a negative feedback on Ctr1B transcription. Consistent with a copper saturation of intestinal cells there is an elevated metallothionein (MtnB) expression. At the same time the lack of Atox1, which is the chaperone that normally escorts copper to the DmATP7 for transport to other parts of the body [39, 40], results in peripheral copper depletion. This scenario also explains the sensitivity of Atox1 mutant flies to copper starvation.

As shown for Atox1-/- mouse fibroblasts [38] we observe a resistance of the Atox1 mutant flies to cisplatin. A major route of cisplatin uptake is via a copper importers of the Ctr type, whereupon these transporters are depleted [30]. Atox1 is required for polyubiquitination, subsequent internalization and proteasomal degradation of Ctr1 upon cisplatin exposure in mouse fibroblasts [38]. As cisplatin is imported via endocytosis of Ctr1 [37], Atox1-/- cells display a resistance to this compound. It is thus tempting to speculate that downregulation of Atox1 may be a mechanism that contributes to cisplatin resistance of some cancer cells.

Acknowledgements

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Figure legends

Figure 1

Generation of the Atox1 null mutant

A) Sequence alignment of copper chaperones that have been shown or suggested to transfer copper ions to ATP7-type transporters. Flybase CG332446 is the fly ortholog of human Atox1. The amino acids that were deleted in the Atox1 mutant allele are underlined.

B) Genomic position of the *Drosophila* Atox1 gene (Flybase CG32446). The deleted region is indicated as a gap. Grey bars represent the neighboring genes, without depicting their exon/intron structure (data from Flybase). The scale bar represents a 1 kb genomic region.

Figure 2

Atox1 mutant flies are sensitive to copper depletion

A) Atox1 mRNA levels were measured in control, EY15780 and $Atox^{174}$ larvae. Controls were also subjected to copper load (250 μM $CuSO_4$) and copper starvation (100 μM chelator BCS). The low expression in EY15780 larvae with a P-element in the promoter of *Atox1* identifies this allele as a hypomorph, while transcripts in the deletion ($Atox1^{174}$) are at background level.

B) Atox1 mutant flies show reduced survival under standard growth conditions (black bar), and cannot develop at all on food containing a copper chelator (250 μM BCS; grey bar). Resistance to BCS was restored by introducing a genomic Atox1 rescue construct into the Atox1 deletion mutant.

Figure 3

Enterocyte Atox1 protein levels are reduced upon copper load

Under standard food condition (NF), Atox1-mCherry shows a punctate staining in the guts of adult flies. When flies are kept on food containing 1 mM $CuSO_4$ for 3 days, staining intensity in enterocytes is substantially reduced. The right panel illustrates the locations where the images were recorded. Close to the basal membrane of adult gut, there are both enterocytes and putative intestinal stem cells, whereas in the middle region, only enterocytes are observed. (Arrows and arrowheads indicate the staining in the enterocytes and in intestinal stem cells, respectively).

Figure 4

Aberrant expression of copper importer Ctr1B in the Atox1 mutant

A) Transcripts of Ctr1B were quantified by the S1 nuclease protection assay. In control animals, Ctr1B expression is induced in the larval gut when they are grown in food with 100 μM BCS. However, in the Atox1 mutant background, the Ctr1B transcript levels were not significantly increased.

B) Atox1 mutant flies show an upregulation of a metallothionein reporter gene (MtnB-EYFP) on NF due to accumulation of intracellular copper, which is not seen in Atox1 heterozygous flies (Atox174/TM2 y+). Autofluorescence of ingested yeast in the gut (orange) was visualized by overlaying the images of FITC and TRITC channels. CC: copper cell region; IC: “iron cell” region. “Iron cells” are specialized metal storing cells which preferentially accumulate iron and copper from the food.

Figure 5

Atox1 mutant flies are more resistant to cisplatin treatment than controls

Compared to control flies, which show decreased survival rate when grown on 1 mM and 1.5 mM cisplatin, Atox1 mutant flies are less sensitive to cisplatin treatment. The P-value was calculated using the student’s t-test.

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Figure 1

A

```
H.sapiens Atox1          ---MPKHEFSVDMTCGGCAEAVSRVLNKLGG--VKYDIDLPNKKVCIESEHSMDTLLATLKKTGKTVSYLGLE-
S.cerevisiae Atx1        MAEIKHYQFNVVMTCSGCSGAVNKVLTKLEPDVSKIDISLEKQLVDVYTTLPYDFILEKIKKTGKEVRSGKQL-
D.melanogaster CG32446   ---MTVHEFKVEMTCGGCASAVERVLGKLGDKVEKVNINLEDRTVSVTSNLSSDELMEQLRKTGKSTTYVGVKK
conservation               :  ::*.* ***.**: **.:** **     * :*.* .: * : :  . * ::  ::**** .
```

96

B

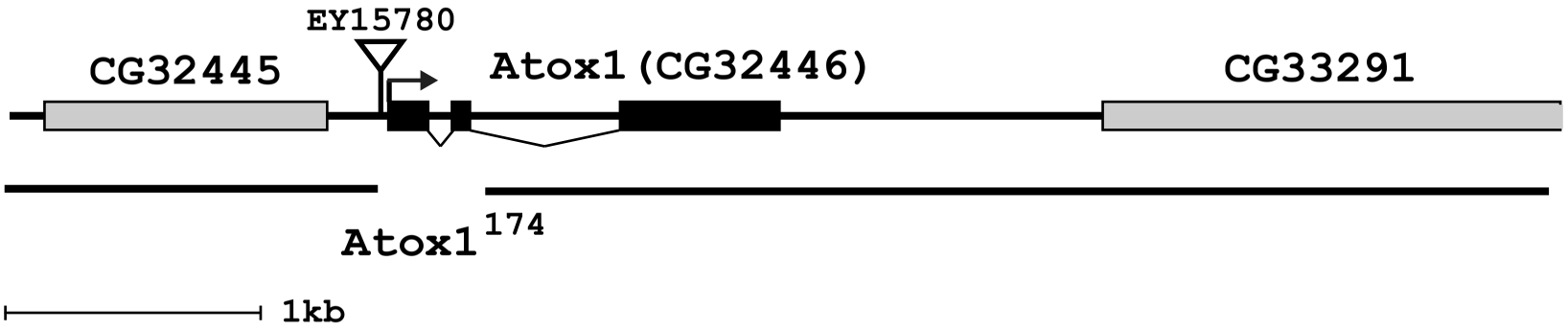
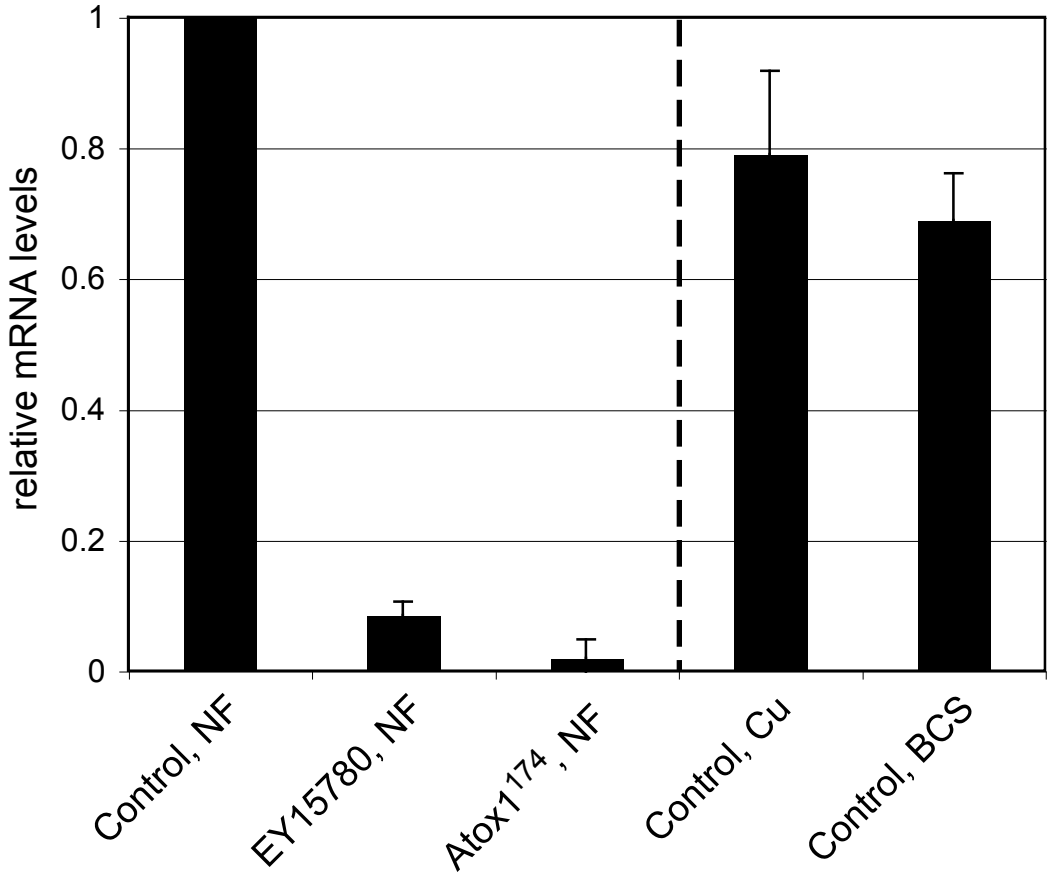


Figure 2

A


relative mRNA levels
1
0.8
0.6
0.4
0.2
0
Control, NF
EY15780, NF
Atox1174 , NF
Control, Cu
Control, BCS

B

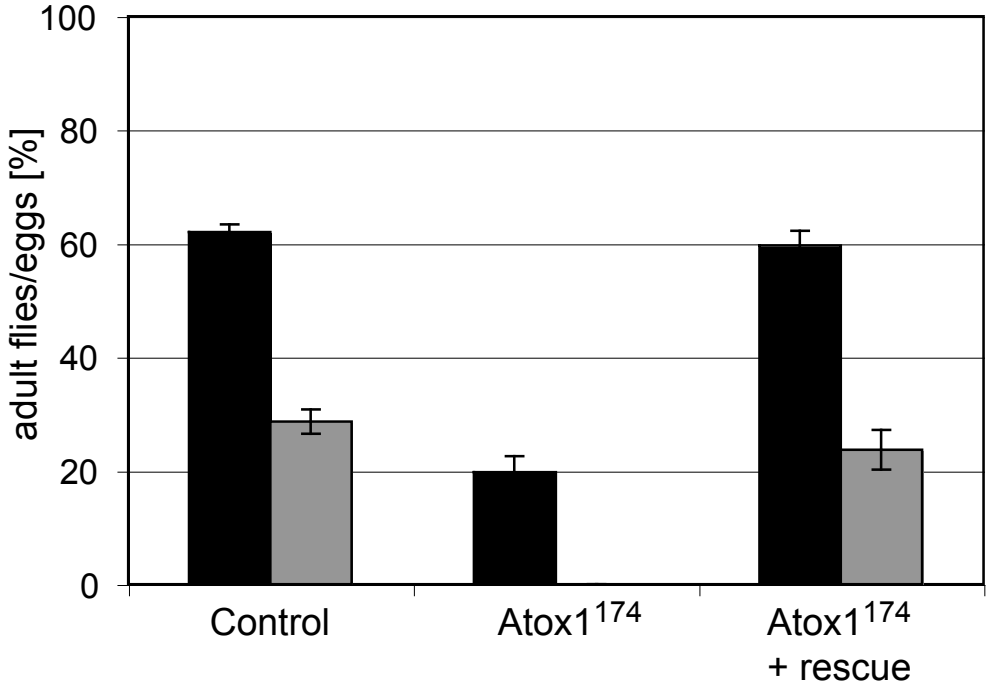

adult flies/eggs [%]
100
80
60
40
20
0
Control
Atox1174
Atox1174
+ rescue

Figure 3

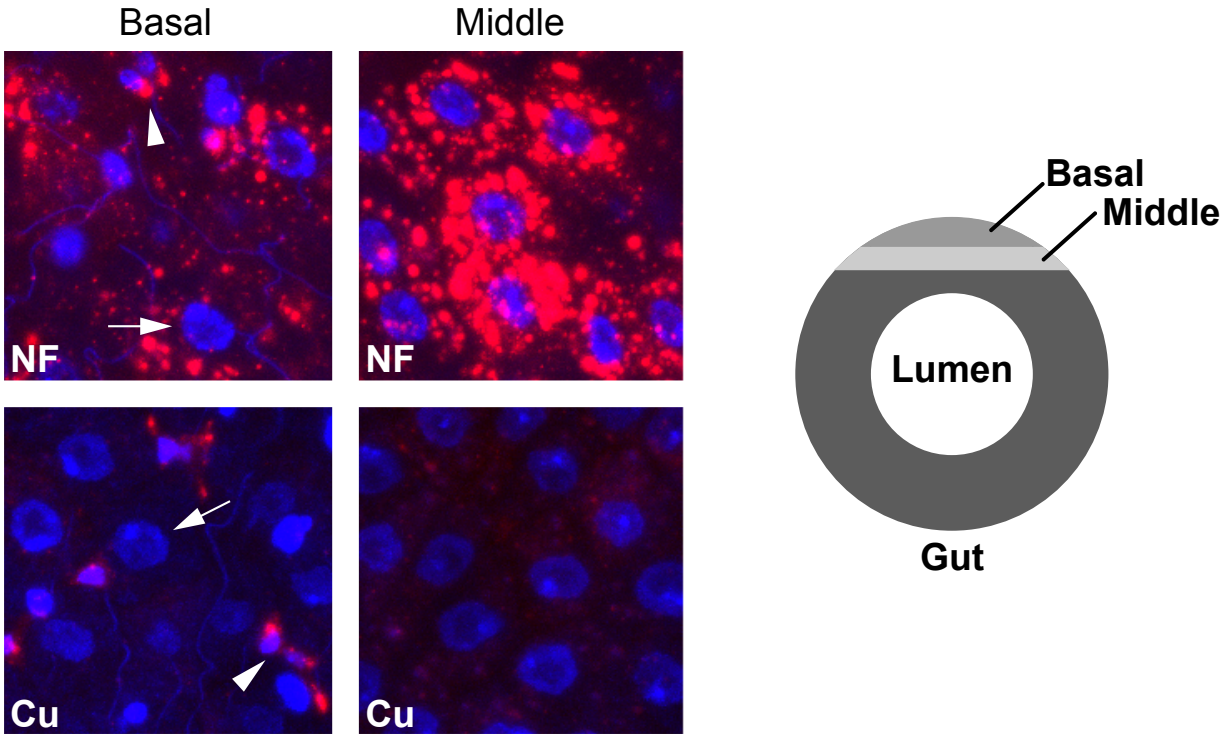
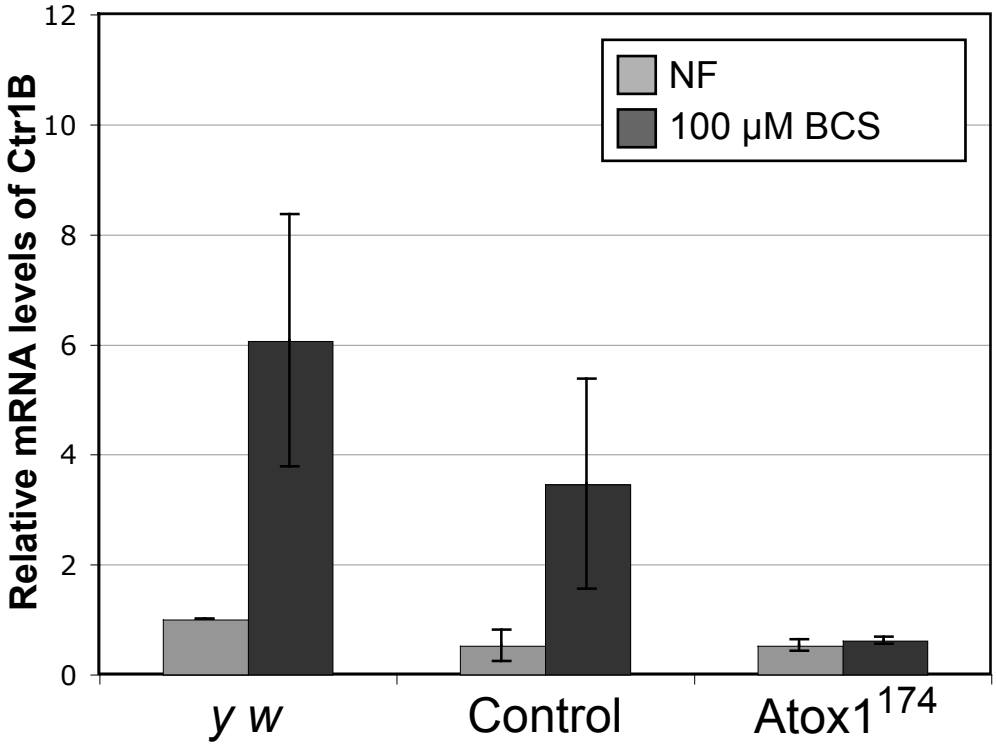


Figure 4

A


Relative mRNA levels of Ctr1B
12
10
8
6
4
2
0
NF
100 µM BCS
y w
Control
Atox1174

B

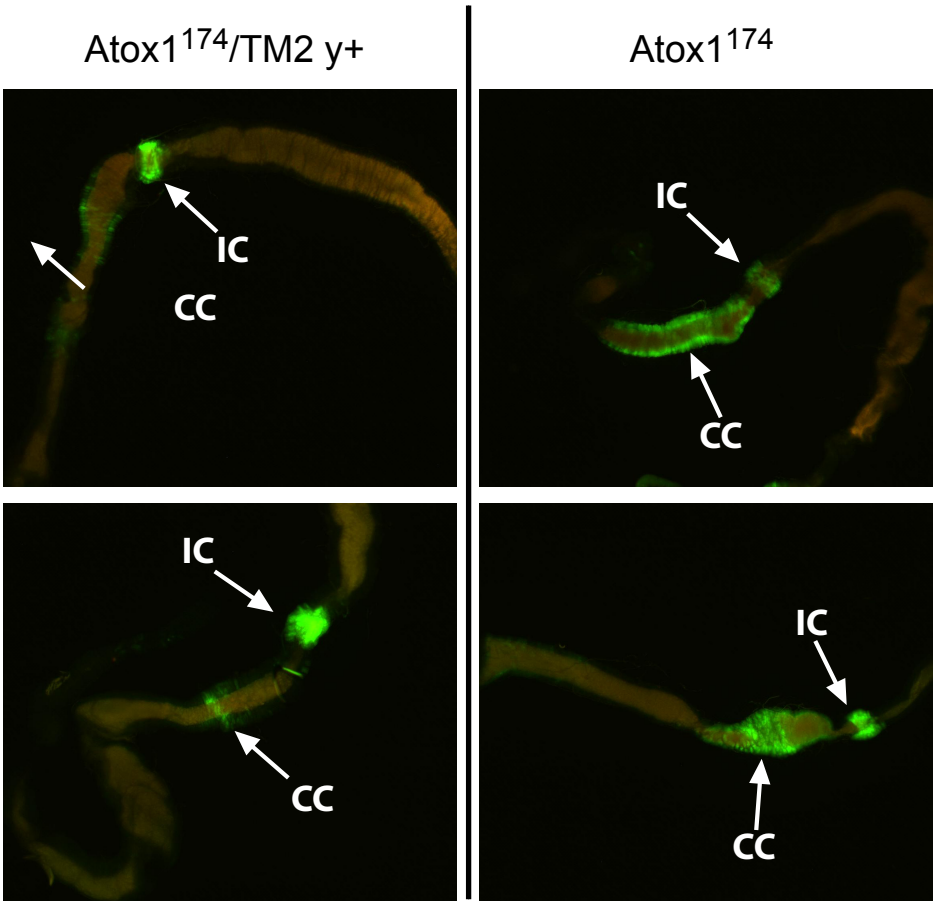
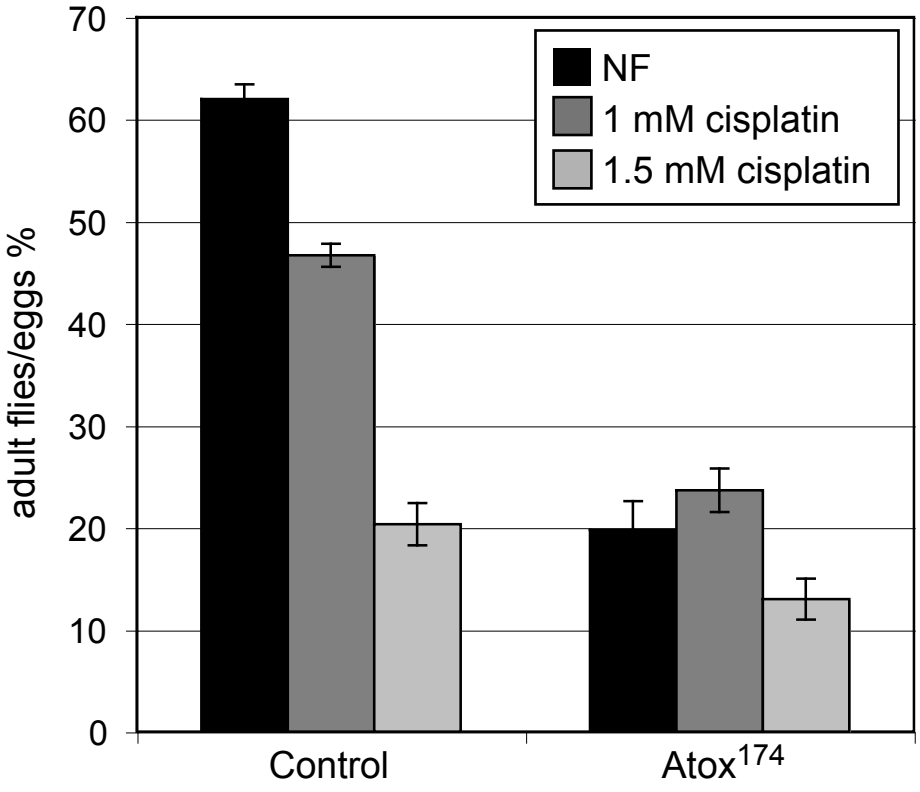

Atox1174/TM2 y+
Atox1174
IC
CC
IC
CC
IC
CC
IC
CC

Figure 5


70
60
50
40
30
20
10
0
adult flies/eggs %
NF
1 mM cisplatin
1.5 mM cisplatin
Control
Atox174

Conclusions and perspectives

A powerful approach to uncover mechanisms underlying fundamental cellular pathways is to compare these pathways and the participating proteins in evolutionary divergent organisms. Over the past years a complex network of proteins, including heavy metal transporters, transcription factors and metallothioneins, involved in heavy metal homeostasis in mammals was discovered. A central player in this network is the metal-responsive transcription factor-1 (MTF-1), which is indispensible for the expression of metallothioneins that bind and thereby scavenge heavy metals. *Drosophila melanogaster* has emerged in the recent years as a good model organism to study heavy metal homeostasis and detoxification. The data presented here shed more light on the functionality and regulation of both human and *Drosophila* MTF-1 and comparing the insights obtained for mammalian tissue cell culture and *Drosophila* will allow a deeper understanding of the steps that are needed to provide an organism with the critical amount of essential heavy metals and protecting against toxic concentrations of trace metals.

In this work we study a conserved cysteine cluster of the mammalian homolog of MTF-1. Mutation of these cysteines results in a strongly decreased transcriptional activity of MTF-1, which is most obvious under conditions of metal exposure. We find that these cysteines mediate homodimerization of MTF-1 and thereby describe a novel protein-protein interaction domain. Zinc, which is an efficient inducer of MTF-1 activity, does not further enhance dimerization. Together with the fact that also the basal level of MTF-1 activity is decreased for the cysteine cluster mutant, we propose that the cysteine cluster is not directly participating in metal sensing. Copper, which induces MTF-1 only slightly, is able to oxidize the cysteines to form intermolecular disulfide bridges and

thereby is stabilizing the dimer. This covalently linked MTF-1 dimer is more sensitive to the induction by zinc. Thereby copper and zinc can synergize to activate metallothionein transcription. This copper effect could not be mimicked by other reagents that induce oxidative stress, like menadione and l-buthionine-sulfoximine (BSO). We consider it likely that these reagents might have a lesser stability in tissue cell culture or do not reach the same cellular compartments as copper and thus are not able to oxidize MTF-1.

It is still not clear how MTF-1 binds to DNA as a dimer since no conserved spacing of inverted or direct repeats is found in MTF-1 target genes. It is possible that the zinc finger domain of the second MTF-1 molecule is not bound to DNA and might even serve as a protein-protein interface to recruit other transcription factors. Otherwise the second partner could bind to neighboring MREs and induce DNA-looping.

Interestingly, several other transcription factors that are involved in metal homeostasis in lower eukaryotes, like Cuf1 of *S. pombe* or Mac1 of *S. cerevisiae*, also contain clusters of cysteines. These clusters must be the result of convergent evolution as they serve different functions, show a different spacing of cysteines and are found in distinct, non-related transcription factors. In contrast to our results obtained for hMTF-1, most of these clusters are in some way involved in metal sensing due to their ability to bind metal ions.

Drosophila MTF-1 lacks the "CQCQCAC" motif of mammalian MTF-1, but contains a differently spaced cluster of cysteines that was shown to mediate copper induced metallothionein transcription. In contrast to hMTF-1, basal activity and activation of the Ctr1B copper importer by starvation are not lost in the dMTF-1 cysteine cluster mutant (2). A transcriptional response to low concentrations of any kind of metal was not found for mammalian MTF-1 and this function might be unique for dMTF-1. It would be

interesting to see if dMTF-1 is also dimerizing. So far, we were not successful in testing dMTF-1 dimerization in a co-immunoprecipitation assay as the dMTF-1 protein is highly unstable and tends to aggregate if overexpressed either in mammalian or *Drosophila* cell culture, which falsifies the obtained results.

Further studies concerning the domain structure of dMTF-1 (part III) should help to reveal the regulatory mechanisms of dMTF-1 and might explain the observed functional differences between human and *Drosophila* MTF-1. A wing phenotype as found for the truncation mutants has not been described in *Drosophila* with altered metal homeostasis so far and might point to a developmental pathway in which dMTF-1 is involved. In line with this, a developmental role of MTF-1 was shown in mice, where null mutants die during embryonic development due to hepatocyte degeneration (10).

Another step of hMTF-1 regulation is discussed in part II of this work. We show that hMTF-1 is acetylated by the transcriptional co-activator p300 and potential acetylation sites could be determined. Histone deacetylase inhibitors boost MTF-1 activity, which suggests that MTF-1 is acetylated in the active state. Further studies might reveal if this acetylation is needed to recruit interacting proteins. As recombinant MTF-1 can bind to DNA it is unlikely that acetylation is needed to support DNA binding.

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